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**WO 01/27132 A1**

(54) Title: ESTRADIOL CONJUGATES AND USES THEREOF

(57) Abstract: A conjugated prodrug of an estradiol compound conjugated to a biological activity modifying agent.

## ESTRADIOL CONJUGATES AND USES THEREOF

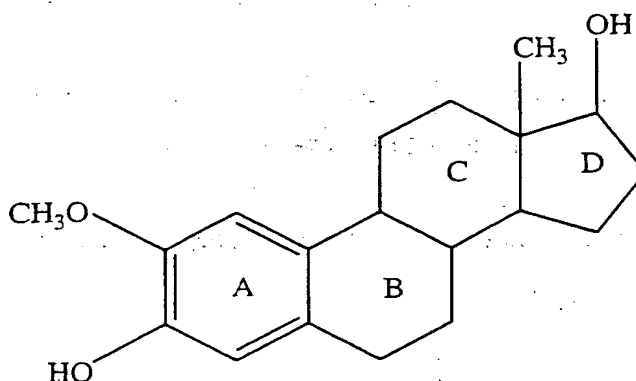
The present invention relates to conjugated prodrugs and uses thereof. More particularly, the invention relates to conjugates of estradiol compounds such as 2-methoxyestradiol and functionally active analogues and derivatives thereof, and the use of such conjugates in the prophylaxis or treatment of conditions associated with enhanced angiogenesis or accelerated cell division, such as cancer, and inflammatory conditions such as asthma and rheumatoid arthritis and hyperproliferative skin disorders including psoriasis. The invention also relates to compositions including the prodrugs of the present invention and methods of synthesizing the prodrugs.

Angiogenesis is crucial for the growth of solid tumors. Without angiogenesis tumour growth is limited to a microscopic size as a result of the limited distances that oxygen and other nutrients may diffuse. Solid tumors produce angiogenic factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), at an early point in development. VEGF is considered to play a more significant role in the initiation of tumour neovascularisation, whereas basic Fibroblast growth factor (bFGF) is considered important in the maintenance of the angiogenic response. There is unequivocal evidence that targeting angiogenesis even after activation of the angiogenic switch can reduce further tumour growth. A further and unanticipated effect of inhibition of tumour angiogenesis inhibition is the reactivation of the host tumour immune response as the non-angiogenic endothelium is more readily stimulated to increase its adhesiveness for lymphocytes, increasing lymphocyte tumour infiltration.

2-methoxyestradiol is an anti-angiogenic, anti-proliferative cytotoxic and anti-inflammatory estradiol metabolite. The formation of catecholestrogens has been regarded as an etiological factor in the association between estradiol levels and breast cancer. The 4-hydroxy- estradiol metabolite is carcinogenic. However, epidemiological evidence suggests that there may be an inverse relationship between 2-Methoxyestradiol forming capacity (catechol-O-methyl transferase) and

risk of breast cancer. In addition, 2-hydroxy-estradiol does not appear to be carcinogenic and is effectively converted into 2-methoxyestradiol. 2-Methoxyestradiol has anti-proliferative effects in a variety of cell types including endothelial cells, fibroblasts, smooth muscle, granulosa cells, Chinese hamster  
5 cell lines and T lymphocytes. In addition, 2-Methoxyestradiol decreases tumour growth and shows activity in a model of arthritis provoked by intra-articular type II collagen injection. 2-Methoxyestradiol has been suggested to be of value in both atherosclerosis and rheumatoid arthritis, each of which is associated with abnormal cell division and angiogenesis.

10. 2-methoxyestradiol is otherwise known as 1,3,5(10)-estratriene-3,17 $\beta$ diol 2 methyl ether and has the following chemical formula:



2-METHYOXYESTRADIOL

It is produced in a two-step process, involving hydroxylation of estrogen to  
15 produce a catecholestrogen followed by methoxylation to produce the corresponding methoxyoestrogen by an inducible cytochrome p450 pathway. The plasma concentrations of 2-methoxyestradiol are not well characterised, in molar levels of approximately 60 pM are detected and in pregnant females levels increase to 30nM. Amounts in urine suggest that 2-methoxyestradiol is present in  
20 considerably lower amounts than the corresponding 2-methoxyestrone which reaches concentrations as high as 10ng/ml of plasma during pregnancy.

D'Amato (WO 95/04535) discloses use of 2-methoxyestradiol and certain

analogues in the treatment of angiogenesis related diseases. Stewart (PCT/AU97/00286) discloses 2-methoxyestradiol and analogues in the treatment of diseases characterised by airway inflammation. However, significant practical limitations are encountered with 2-methoxyestradiol: it has dose-limiting toxicity within the therapeutic range. It is highly insoluble in aqueous solution and therefore difficult to administer.

It is an object of the present invention to overcome, or at least alleviate, one or more of the difficulties or deficiencies associated with the prior art.

In one aspect, the present invention provides a conjugated prodrug of an estradiol compound, preferably of 2-methoxyestradiol or a functionally active analogue or derivative thereof, conjugated to a biological activity modifying agent.

Preferably, the biological activity modifying agent also functions as a masking and/or targeting agent.

The biological activity modifying agent is preferably a carbohydrate or peptide moiety.

By "functionally active" is meant that the analogue or derivative of 2-methoxyestradiol has one or more of the biological activities of 2-methoxyestradiol. The biological activities of 2-methoxyestradiol include, but are not limited to: inhibition of endothelial cell proliferation; inhibition of smooth muscle cell proliferation; inhibition of tumour cell proliferation inhibition of microtubule function; inhibition of leukocyte activation. Examples of such functionally active analogues or derivatives include 2-ethoxyestradiol, 2-hydroxyestradiol and other analogues modified at the 2 position, 2methoxyestradiol-3methylether, 4-methoxyestradiol, and other analogues in which the B ring is expanded to a 7-numbered ring. See also WO95/04535, the entire disclosure of which is incorporated herein by reference.

Preferably the conjugated prodrug includes 2-methoxyestradiol or a functionally active analogue or derivative thereof, conjugated to a sugar moiety.

Preferably the sugar moiety is a substrate for an enzyme which is likely to be present, or present in increased levels, in the condition to be treated. In this way, the compound will have a servo-controlled bioavailability, in that the level of enzyme is a determinant of the rate at which the drug (an estradiol compound such as 2-methoxyestradiol or functionally active analogue or derivative thereof),  
5 is released from the prodrug. This also enables localised release of the drug, for example at the site of a solid tumour.

More preferably the sugar moiety is a 5- or 6-membered sugar. A particularly preferred sugar moiety is a glucuronide, which is a substrate for glucuronidase, a tumour associated enzyme, also likely to be present in  
10 inflammatory responses. Another particularly preferred sugar moiety is a galactoside, which is a substrate for the enzyme galactosidase. Neoplastic and inflammatory diseases are associated with elevated expression of  $\beta$ -galactosidase and other lysosomal hydrolytic enzymes. These may convert pro-drugs to active  
15 agents at a rate which is proportional to the extent and activity of disease. This novel drug targeting mechanism results in lessening of systemic toxicity. The use of a glucuronide or galactoside conjugate of the estradiol compound such as 2-methoxyestradiol or its analogues also renders the compound water soluble and therefore increases the ease of formulation for any of the desired routes of  
20 administration.

Alternatively, the conjugated prodrug according to the present invention includes 2-methoxyestradiol or a functionally active analogue or derivative thereof, conjugated to a peptide moiety.

More preferably this peptide moiety is a tri-, tetra- or higher peptide. For  
25 example, this peptide may be, or include the sequence -Ala-Phe-Lys or -Val-Leu-Lys.

The incorporation of an estradiol compound such as 2-methoxyestradiol into a disease-dependently activated pro-drug enables significant improvement of potency and selectivity of this anti-cancer and anti-inflammatory agent. Two  
30 features of disease activity are exploited by a prodrug bearing, for example, a

galactoside group linked directly or via a linker to the parent molecule.  $\beta$ -galactosidase is upregulated in senescent endothelial cells and in macrophages that have differentiated from monocytes. Endothelial senescence is expected in areas of active angiogenesis (local endothelial cells having undergone many population doublings) and where there is significant macrophage infiltration. Both of these conditions are met in chronic inflammation in rheumatoid arthritis and in development of solid tumours. Endothelial cells proliferating to neovascularise tumours will show higher proportions of senescent cells that will express increased senescence-associated  $\beta$ -galactosidase. This expression, in addition to that resulting from release of leukocyte lysosomal hydrolases, serves to localise the conversion of 2-methoxyestradiol-17 $\beta$ -galactopyranoside. Macrophages are known to acquire increased expression of lysosomal  $\beta$ -galactosidase upon further differentiation induced by cytokines and phagocytic stimuli. Thus, macrophage-dependent activation of the pro-drug may also occur. 2-Methoxyestradiol itself induces an increase in expression of  $\beta$ -galactosidase in endothelial cells, and thus incubation of 2-methoxy-3,17 $\beta$ -estradiol-beta-D-galactopyranosides with endothelial cells produces a positive feedback in activation of pro-drug.

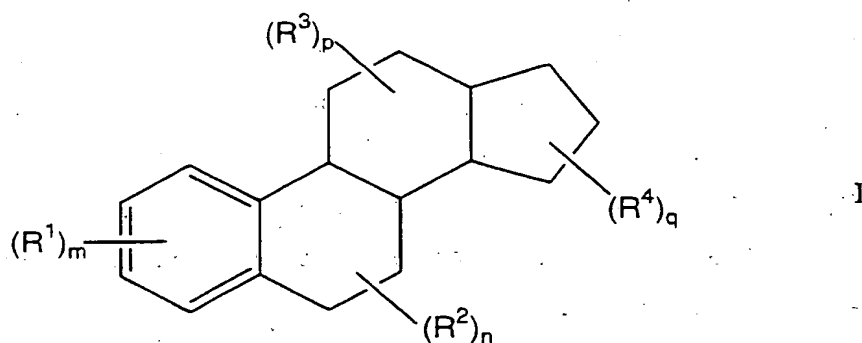
The sugar protein or peptide moiety may be conjugated to the estradiol compound at any suitable position. Preferably, the sugar, protein or peptide moiety is conjugated to the estradiol compound such as 2-methoxyestradiol or its analogues at positions 3 and/or 17.

In a particularly preferred form of this aspect of the invention, the conjugated prodrug is 17 $\beta$  galactopyranoside-2-methoxyestradiol or a functionally active analogue or derivative thereof. A galactoside conjugate of 2-methoxyestradiol has been found to have improved aqueous solubility and less cytotoxicity than the parent, 2-methoxyestradiol.

The sugar may be conjugated directly to the estradiol compound such as 2-methoxyestradiol or its analogue, or the two may be conjugated via a cleavable link. The cleavable link may be of any suitable type, for example a carbonate or carbamate. A suitable link may be readily determined by those skilled in the art.

Use of a link may have the advantage of stabilizing the prodrug in the plasma, for example.

In a further aspect of the present invention, there is provided a compound having the formula I



5

wherein m, n, p and q are each integers of 0, 1, 2 or 3,

each R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup> and R<sup>4</sup> is a substituent independently selected from one or more of hydrogen, hydroxy, halo, optionally substituted C<sub>1-6</sub> alkyl, C<sub>1-6</sub> alkenyl, C<sub>1-6</sub> alkynyl, C<sub>3-8</sub> cycloalkyl, C<sub>1-6</sub> alkanoyl, C<sub>1-6</sub> alkoxy, aryl, amino, acyl, C<sub>1-6</sub> acyloxy aminoalkyl, imino, C<sub>1-6</sub> iminoalkyl, C<sub>1-3</sub> imino cycloalkyl, keto, aldehyde, hydrazono, oximino, hydroximino, C<sub>1-6</sub> alkoximino, C<sub>3-8</sub> cycloalkoximino or carboxyl groups, or an O-Protecting group, or a carbohydrate, peptide or protein residue, or derivative thereof or a pharmaceutically acceptable salt or in vivo hydrolysable ester, amide carbonate, or carbamate thereof:

15

with the proviso that at least one of R<sup>1</sup> to R<sup>4</sup> is present.

The compounds described above are compounds which may function as conjugated prodrugs, as described herein and/or may function as intermediates in the preparation of such conjugated prodrugs.

Any substituent may be optionally substituted, by one or more substituents, which may be the same or different. Typical substituents include: hydroxy; C<sub>1-6</sub>alkoxy for example methoxy; mercapto; C<sub>1-6</sub>alkylthio for example methylthio; amino; C<sub>1-6</sub>alkylamino, for example methylamino; di-(C<sub>1-6</sub>alkyl)amino, for example dimethylamino; carboxy; carbamoyl; C<sub>1-6</sub>alkylcarbamoyl, for example methylcarbamoyl; di-C<sub>1-6</sub>alkylcarbamoyl for example dimethylcarbamoyl; C<sub>1-6</sub>alkylsulphonyl, for example methylsulphonyl; arylsulphonyl, for example phenylsulphonyl; C<sub>1-6</sub>alkylaminosulphonyl, for example methylaminosulphonyl; di-(C<sub>1-6</sub>alkyl)aminosulphonyl, for example dimethylamino-sulphonyl; nitro; cyano; cyano-C<sub>1-6</sub>alkyl, for example cyanomethyl; hydroxyC<sub>1-6</sub>alkyl, for example hydroxymethyl; amino-C<sub>1-6</sub>alkyl, for example aminoethyl; C<sub>1-6</sub>alkanoyl-amino, for example acetamido; C<sub>1-6</sub>alkoxycarbonylamino, for example methoxycarbonylamino; C<sub>1-6</sub>alkanoyl, for example acetyl; C<sub>1-6</sub>alkanoyloxy, for example acetoxy; C<sub>1-6</sub>alkyl, for example methyl, ethyl, isopropyl or tert-butyl; halo, for example fluoro, chloro or bromo; trifluoromethyl or trifluoromethoxy.

Preferably each of R<sup>1</sup> to R<sup>4</sup> is selected from hydrogen hydroxy, halo, optionally substituted C<sub>1-6</sub> alkyl, C<sub>1-6</sub> alkoxy, imino, C<sub>1-6</sub> iminoalkyl, C<sub>3-8</sub> iminocycloalkyl, keto, aldehyde, hydroximino alkoximino, hydrazono, carbamate, an O-Protecting group, or a sugar protein or peptide residue.

More preferably each of R<sup>1</sup> to R<sup>4</sup> is selected from hydroxy, C<sub>1-6</sub> alkoxy, imino C<sub>3-8</sub> iminocycloalkyl, keto, hydrazono, carbamate, or a sugar, protein, or peptide residue.

Most preferably at least one of R<sup>1</sup> to R<sup>4</sup> is a carbohydrate, peptide or protein residue or derivative thereof, or a keto, hydrazono or carbamate group.

Particularly preferred compounds according to the present invention include compounds wherein R<sup>1</sup> is one or more of hydroxy, alkoxy, acyloxy or a carbohydrate, peptide or protein residue;

R<sup>3</sup> includes an alkyl group; and

R<sup>4</sup> is one or more of hydroxy, alkoxy, O-Protecting groups or a carbohydrate, protein or peptide residue.





Other compounds which may be used as conjugated prodrugs include compounds in which one of  $R^1$ ,  $R^2$  or  $R^4$  is a peptide residue having the formula III:



5 wherein Z is a spacer or bond

m, n and p are each integers of 0, 1 or 2

ABCDFGHJK, which may be the same or different, are each amino acids,

Where the biological activity modifying agent is a peptide moiety, the conjugated prodrug may be synthesised utilising techniques similar to those described in Franciscus et al (Franciscus et al, 2000), the entire disclosure of which is incorporated herein by reference.

Specific examples of preferred compounds according to the present invention include:

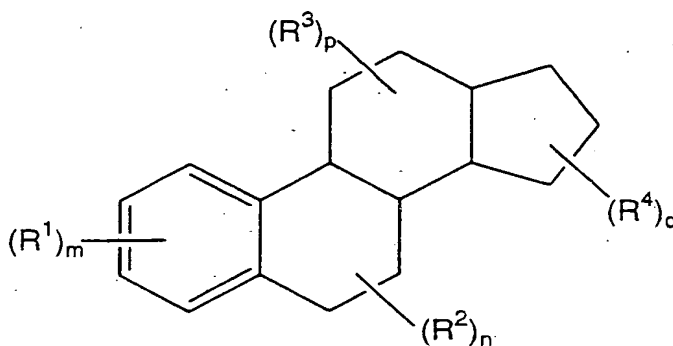
- 3-Acetyl 2-methoxyestradiol
- 15 3-Acetyl 6-oxo-2-methoxyestradiol
- 3,17-Diacetyl-2-methoxyestradiol
- 17-Acetyl-2-methoxyestradiol
- 3,17-Diacetyl-6-oxo-2-methoxyestradiol
- 3,17-Diacetyl-2-methoxyestradiol-6-hydrazone
- 20 2-Methoxyestradiol-6-hydrazone
- 2-Methoxy estradiol-17 $\beta$ -monodienyl ether
- 2-Methoxy estradiol-17 $\beta$ -acetate
- 2-Methoxy-17 $\beta$ -Estradiol-17-D-triacetylglucopyranosiduronic acid methyl ester
- 25 2-Methoxy-17 $\beta$ -Estradiol-17-D-glucopyranosiduronic acid
- 2,3,17 $\beta$ -estradiol-17- $\beta$ -D-galactopyranoside-2-methyl ether
- 2,3,17 $\beta$ -estradiol-3- $\beta$ -D-galactopyranoside-2-methyl ether.

Suitable pharmaceutically acceptable salts include acid addition salts such

as hydrochloride, hydrobromide, citrate tartrate and maleate salts and salts formed with phosphoric and sulphuric acid. In another aspect suitable salts are base salts such as an alkali metal salt for example sodium or potassium, an alkaline earth metal salt for example calcium or magnesium, or organic amine salt for example triethylamine.

In vivo hydrolysable esters, amides, carbonates and carbamates hydrolyse in the human body to produce the parent compound. Such esters, amides, carbonates and carbamates can be identified by administering, for example intravenously to a test animal, the compound under test and subsequently examining the test animal's body fluids. Suitable in vivo hydrolysable groups include N-carbomethoxy and N-acetyl.

Accordingly, in a further aspect of the present invention, there is provided a conjugated prodrug of the formula:



I

wherein  $m$ ,  $n$ ,  $p$  and  $q$  are each integers of 0, 1, 2 or 3, each  $R^1$ ,  $R^2$ ,  $R^3$  and  $R^4$  is substituent independently selected from one or more of hydrogen, hydroxy, halo, optionally substituted  $C_{1-6}$  alkyl,  $C_{1-6}$  alkenyl,  $C_{1-6}$  alkynyl,  $C_{3-8}$  cycloalkyl,  $C_{1-6}$  alkanoyl,  $C_{1-6}$  alkoxy, aryl, amino, acyl,  $C_{1-6}$  acyloxy aminoalkyl, imino,  $C_{1-6}$  iminoalkyl,  $C_1$ - $C_3$  imino cycloalkyl, keto, aldehyde, hydrazono, oximino, hydroximino,  $C_{1-6}$  alkoximino,  $C_{3-8}$  cycloalkoximino or carboxyl groups, or an O-Protecting group, or a carbohydrate, peptide or protein residue, or derivative thereof or a pharmaceutically acceptable salt or in vivo hydrolysable ester, amide carbonate, or carbamate thereof:

with the proviso that at least one of  $R^1$  to  $R^4$  is present and includes a carbohydrate, protein or peptide residue.

Preferably the sugar residue is a glucuronide or galactoside, as described above. Preferably the peptide residue is, or includes the sequence D-Ala-Phe-Lys or D-Val-Leu-Lys.

5 In a still further aspect of the present invention there is provided a method of prophylaxis or treatment of a condition associated with angiogenesis or accelerated or increased amounts of cell division hypertrophic growth or inflammation, said method including administering to a patient in need of such prophylaxis or treatment an effective amount of a conjugated prodrug according to the present invention, as described above.

10 It should be understood that prophylaxis or treatment of said condition includes amelioration of said condition.

By "an effective amount" is meant a therapeutically or prophylactically effective amount. Such amounts can be readily determined by an appropriately skilled person, taking into account the condition to be treated, the route of  
15 administration and other relevant factors. Such a person will readily be able to determine a suitable dose, mode and frequency of administration.

The pharmaceutical compositions of this invention will normally be administered to humans so that, for example, a daily dose of 0.05 to 300, preferably 150, more preferably 75 mg/kg body weight (and preferably of 0.1 to 30  
20 mg/kg body weight) is received. This daily dose may be given in divided doses as necessary, the precise amount of the compound received and the route of administration depending on the weight, age and sex of the patient being treated and on the particular disease condition being treated accordingly to principles known in the art.

25 Typically unit dosage forms will contain about 1 mg to 500 mg of a compound of the present invention.

The method of the present invention may be used to prevent, ameliorate or treat inflammatory conditions, particularly conditions characterised by chronic or

acute airway inflammation, such as asthma or chronic inflammatory conditions, such as rheumatoid arthritis. The method of the present invention may also be used to treat any conditions associated with enhanced angiogenesis or accelerated cell division, particularly cancers, such as solid tumours.

- 5           The patient to be treated is preferably a human, although the method of the present invention may also be used to treat other mammals such as cats, dogs, horses, sheep, pigs and cattle.

          In a further aspect of the present invention there is provided a pharmaceutical composition comprising a conjugated prodrug according to the  
10       present invention, together with a pharmaceutically acceptable carrier, diluent or excipient.

          The pharmaceutical composition may be used for the prophylaxis or treatment of conditions associated with angiogenesis or accelerated cell division or inflammation.

- 15           The pharmaceutical compositions including the prodrug of the present invention may be administered in standard manner for the disease condition that it is desired to treat, for example by oral, topical, parenteral (eg. intravenous, subcutaneous or intramuscular), buccal, nasal, vaginal or rectal administration or by inhalation. For these purposes the compounds of this invention may be  
20       formulated by means known in the art into the form of, for example, tablets, capsules, aqueous or oily solutions, suspensions, emulsions, creams, ointments, gels, nasal sprays, suppositories, finely divided powders or aerosols for inhalation, and for parenteral use (including intravenous, intramuscular or infusion) sterile aqueous or oily solutions or suspensions or sterile emulsions. A preferred route of  
25       administration is intravenously in sterile isotonic solution.

          In the treatment of inflammatory airway conditions such as asthma, the prodrug may be administered by routes including but not limited to topical, oral, nasal and inhalation.

In addition to the compounds of the present invention, the pharmaceutical composition of this invention may also contain, or be co-administered (simultaneously or sequentially) with, one or more pharmacological agents of value in treating one or more disease conditions referred to hereinabove.

5           In addition, the prodrug may be incorporated into biodegradable polymers allowing for sustained release, the polymers being implanted in the vicinity of where delivery is desired, for example, at the site of a tumour. The biodegradable polymers and their use are described in detail in Brem et al., *J. Neurosurg.* 74:441-446 (1991).

10           A person skilled in the art will be able by reference to standard texts, such as Remington's Pharmaceutical Sciences 17th edition, to determine how the formulations are to be made and how these may be administered.

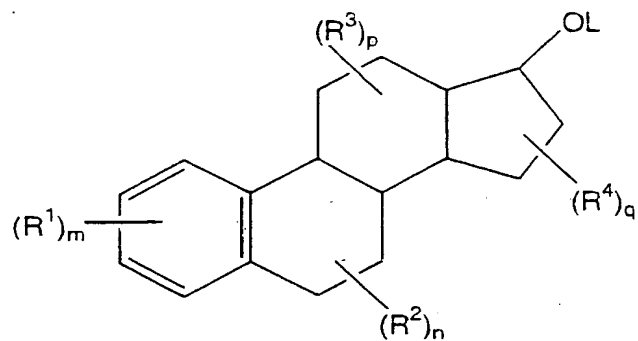
Such formulation techniques include the step of bringing into association the prodrug and the pharmaceutically acceptable carrier(s), diluent(s) or  
15   excipient(s). In general, the formulations are prepared uniformly and intimately bringing into association the prodrug with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

In a further aspect of the present invention there is provided use of a conjugated prodrug according to the present invention for the preparation of a  
20   medicament for the prophylaxis or treatment of conditions associated with angiogenesis or accelerated cell division or inflammation.

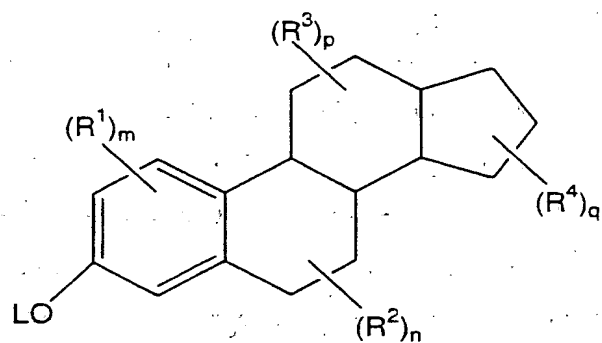
In a still further aspect of the present invention there is provided a method of preparing a compound of formula I as described above which method includes:

(a)       reacting a compound of formula IV, V or VI with a compound of  
25   formula VII or VIII:

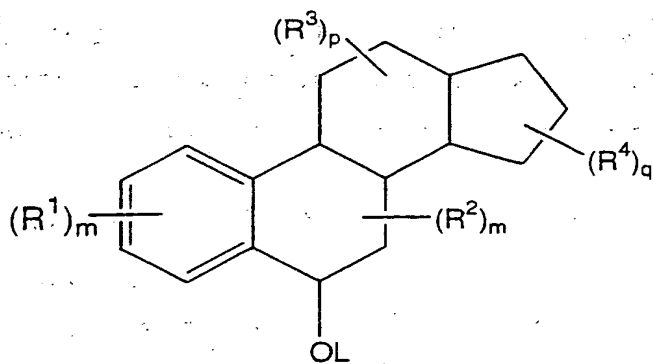
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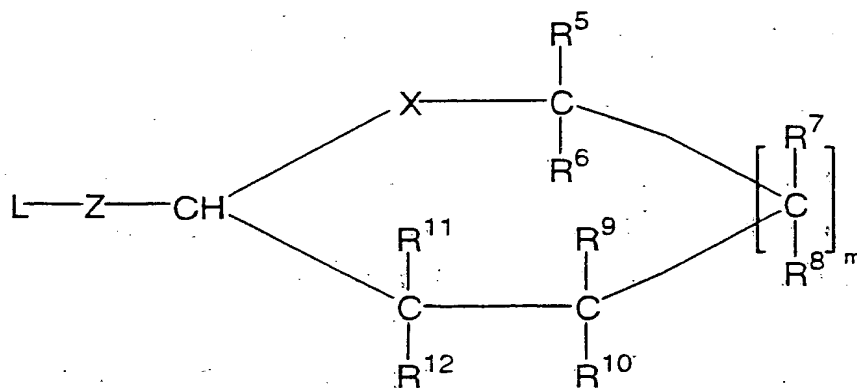
IV



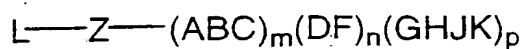
V



VI



VII



VIII

wherein  $R^1$ ,  $R^2$ ,  $R^3$ ,  $R^4$ , ABCDEFGHJK and m, n, p and q are as defined in claim 10 and 18 except that in formula IV,

q is 0, 1 or 2;

in Formula V, m is 0, 1 or 2 and

5 in Formula VI, n is 0, 1 or 2

$R^5$  to  $R^{12}$  are as defined above,

L is a leaving group,

Z is a spacer or bond;

wherein any functional group is protected, if necessary; and

10 (b) removing any protecting groups;

(c) optionally, before or after said reaction converting any compound of formula III, V or VI into another compound of formula IV, V or VI;

(d) optionally forming a pharmaceutically acceptable salt or in vivo hydrolysable ester, amide, carbonate or carbamate.

15 Protecting groups may in general be chosen from any of the groups described in the literature or known to the skilled chemist as appropriate for the protection of the group in question, and may be introduced by conventional methods.

Protecting groups may be removed by any convenient method as described  
20 in the literature or known to the skilled chemist as appropriate for the removal of the protecting group in question, such methods being chosen so as to effect removal of the protecting group with minimum disturbance of groups elsewhere in the molecule.

A carboxyl protecting group may be the residue of an ester-forming  
25 aliphatic or araliphatic alcohol or of an ester-forming silanol (the said alcohol or silanol preferably containing 1-20 carbon atoms).

Examples of carboxy protecting groups include straight or branched chain  
(1-12C) alkyl groups (eg. isopropyl, *t*-butyl); lower alkoxy lower alkyl groups (eg. methoxymethyl, ethoxymethyl, isobutoxymethyl); lower aliphatic acyloxy lower  
30 alkyl groups, (eg. acetoxymethyl, propionyloxymethyl, butyryloxymethyl,



pivaloyloxymethyl); lower alkoxy carbonyloxy lower alkyl groups (eg 1-methoxycarbonyloxyethyl, 1-ethoxycarbonyloxyethyl); aryl lower alkyl groups (eg. benzyl, p-methoxybenzyl, o-nitrobenzyl, p-nitrobenzyl, benzhydryl and phthalidyl); tri(lower alkyl)silyl groups (eg. trimethylsilyl and t-butyl dimethylsilyl); tri(lower alkyl)silyl lower alkyl groups (eg. trimethylsilylethyl); and (2-6C) alkenyl groups (eg. allyl and vinyl ethyl).

Methods particularly appropriate for the removal of carboxyl protecting groups include for example acid, base-, metal- or enzymically-catalysed hydrolysis.

Examples of hydroxyl protecting groups include lower alkyl groups (eg. t-butyl), lower alkenyl groups (eg. allyl); lower alkanoyl groups (eg. acetyl); lower alkoxy carbonyl groups (eg. t-butoxycarbonyl); lower alkenyloxy carbonyl groups (eg. allyloxy carbonyl); aryl lower alkoxy carbonyl groups (eg. benzyloxy carbonyl, p-methoxybenzyloxy carbonyl, o-nitrobenzyloxy carbonyl, p-nitrobenzyloxy carbonyl); tri(lower alkyl)silyl (eg. trimethylsilyl, t-butyl dimethylsilyl) and aryl lower alkyl (eg. benzyl) groups. An acetyl group is preferred.

Examples of amino protecting groups include formyl, aralkyl groups (eg. benzyl and substituted benzyl, p-methoxybenzyl, nitrobenzyl and 2,4-dimethoxybenzyl, and triphenylmethyl); di-p-anisylmethyl and furylmethyl groups; lower alkoxy carbonyl (eg. t-butoxycarbonyl); lower alkenyloxy carbonyl (eg. allyloxy carbonyl); aryl lower alkoxy carbonyl groups (eg. benzyloxy carbonyl, p-methoxybenzyloxy carbonyl, o-nitrobenzyloxy carbonyl, p-nitrobenzyloxy carbonyl); trialkylsilyl (eg. trimethylsilyl and t-butyl dimethylsilyl); alkylidene (eg. methylidene); benzylidene and substituted benzylidene groups.

Methods appropriate for removal of hydroxy and amino protecting groups include, for example, acid-, base-, metal- or enzymically-catalysed hydrolysis, for groups such as p-nitrobenzyloxy carbonyl, hydrogenation and for groups such as o-nitrobenzyloxy carbonyl, photolytically.

Pharmaceutically acceptable salts of the compound of the formula (I) may

be prepared in any conventional manner for example from the free base and acid. In vivo hydrolysable esters, amides and carbamates may be prepared in any conventional manner.

The reaction between the compounds of the formulae IV, V or VI and VII  
5 or VIII is performed in conventional manner. Typically this reaction takes place in organic solvent for example an anhydrous aprotic solvent such as dimethylformamide, dimethylacetamide, tetrahydrofuran, toluene or benzene. The reaction is generally performed in the presence of a catalyst, such as a cadmium salt for example cadmium carbonate, and is generally performed at ambient or  
10 elevated temperature for example 0°-100°C, more preferably 40°-80°C.

In the compounds of the formula IV, V, VI, VII or VIII, L is a conventional leaving group such as a hydrogen ion, halo for example chloro, iodo or bromo; or a tosylate for example p-toluenesulphonyloxy or methanesulphonyloxy.

The compound of formula VII may be a protected sugar derivative,  
15 preferably an acetylated sugar. The acetylated sugar may be of any suitable type. Preferably the acetylated sugar is an acetyl glucuronate or acetyl galactoside.

Compounds of the formula IV, V, VI, VII or VIII which has a protecting group may be deprotected in standard manner. Any suitable N-protecting group may be used and deprotected in conventional manner. Favourably the protecting  
20 group is C<sub>1-6</sub>alkoxycarbonyl and such compounds may be converted to compounds of the formula (I) wherein R is methyl for example by treating with a reducing agent such as lithium aluminium hydride. Certain compounds of the formula (V) are also in vivo hydrolysable esters, amides, carbonates or carbamates of the compounds of the formula (I).

25 Compounds of the formula IV, V or VI wherein R<sup>1</sup> to R<sup>4</sup> is hydrogen may be converted to compounds wherein R<sup>1</sup> to R<sup>4</sup> is other than hydrogen. For example such conversion may comprise conventional methods of alkylation with an appropriate alkylating agent or reductive amination. For example an isopropyl

group may be prepared by reacting a compound wherein R is hydrogen with acetone in the presence of a reducing agent such as sodium borohydride or sodium cyanoborohydride. A 2-methylpropyl group may be prepared by reacting a compound wherein R is hydrogen with isobutyric acid in the presence of a  
5 reducing agent such as sodium borohydride or sodium cyanoborohydride.

Preferably the reaction is conducted in the presence of a catalyst. The catalyst may be of any suitable type. Preferably the catalyst is a cadmium salt such as cadmium carbonate.

The product may be isolated by any suitable technique(s) as will be readily  
10 apparent to an appropriately skilled person. Filtration, flash chromatography and preparative HPLC have been found to be suitable.

The present invention will now be more fully described with reference to the accompanying Examples. It should be understood, however, that the description following is illustrative only and should not be taken in any way as a restriction on  
15 the generality of the invention described above.

#### **Example 1: Synthesis of 2-Methoxy-17 $\beta$ -Estradiol-17-D-glucopyranosiduronic acid**

(See Figure 1)

#### **$\beta$ -Estradiol Dibenzyl Ether (2)**

20  $\beta$ -Estradiol (20.00g, 73.4mmol) was dissolved in 300mL anhydrous DMF and cooled to 0°C under nitrogen. Sodium Hydride (10.4g, 260mmol) was added in portions with constant stirring. Stirring continued at 0°C for a further 15 minutes at which time Benzyl Bromide (40.0mL, 336 mmol) was added dropwise followed by addition of tetrabutylammoniumiodide (2.0g, 5.4mmol). Stirring of reaction then  
25 continued at room temperature for 72 hours. The reaction mixture was cooled to 0°C and excess Sodium hydride quenched with 100mL 50% aqueous Ethanol. The reaction mixture was acidified to pH 3 with 3M HCl solution. Total reaction mixture was then evaporated in vacuo to a volume of approximately 300mL and

extraction was performed with 3 x 150 mL Ethyl Acetate in the presence of saturated Sodium Chloride solution (200mL). Combined extracts were washed with water (200mL), saturated Sodium Chloride solution (200mL), and dried over anhydrous Sodium Sulphate and evaporated in vacuo to give a viscous orange oil.

- 5 Trituration with Petroleum Spirit yielded a cream coloured solid (26.3g 76.8% yield). TLC analysis of the product indicated a single spot.

### 2-Bromo- $\beta$ -Estradiol Dibenzy Ether (3)

- A stirred solution of  $\beta$ -Estradiol Dibenzy Ether (18.60g, 41.1 mmol) in 120mL anhydrous THF/180mL Acetic Acid was cooled below -10°C under  
10 nitrogen. Bromine (2.53 mL, 49.4mmol) was added dropwise over a 30 minute period ensuring temperature remained at or below -10°C. Stirring continued at -10°C for 30 minutes before reaction was warmed to room temperature and stirring continued for a further 2.5 hours. Reaction mixture was poured onto an ice/water mixture (400mL) and extraction performed with 3 x 200mL of Dichloromethane  
15 with combined organic layers being washed with water (200mL), saturated Sodium Bicarbonate solution (200mL), 10% Sodium Thiosulphate solution (200mL) and water (200mL) and dried over anhydrous Sodium Sulphate. Evaporation yielded a gummy cream solid which was recrystallised from Ethyl Acetate to give a cream coloured crystalline solid (13.21 g, 60.5%). TLC analysis indicated a single spot.

### 20 2-Hydroxy- $\beta$ -Estradiol Dibenzy Ether (4)

- A stirred solution of 2-Bromo- $\beta$ -Estradiol Dibenzy Ether (10.00g, 18.8mmol) in 200mL anhydrous THF was cooled to -78°C under nitrogen and a 1.7M solution of t-butyllithium in Pentane (24.35mL, 41.4mmol) was added dropwise ensuring reaction temperature remained below -70°C. Reaction mixture was stirred at this  
25 temperature for 15 minutes at which time MoOPH reagent (9.08g, 20.7mmol) was added dropwise as a suspension in anhydrous THF ensuring reaction temperature remained below -70°C, with stirring continuing at this temperature for a further 1 hour, before reaction was warmed to 0°C and stirred for a further 1 hour. Reaction mixture was quenched dropwise addition of water followed by extraction with 3 x

150mL Dichloromethane. Combined organic layers were washed with 50% water/50% saturated Sodium Chloride solution (150mL), Saturated Sodium Bicarbonate solution (150mL) and saturated Sodium Chloride solution (150mL) prior to being dried over anhydrous Sodium Sulphate. Evaporation yielded the  
5 crude product as a dark green viscous oil. TLC analysis showed an impure product that was purified by flash chromatography to yield 5.56g (63.2%) of the pure product.

### 2-Methoxy- $\beta$ -Estradiol Dibenzy Ether (5)

Anhydrous Potassium Carbonate (12.03g, 87.0mmol) was added to a  
10 stirred solution of 2-Hydroxy- $\beta$ -Estradiol Dibenzy Ether (4.08g, 8.7mmol) in anhydrous DMF (250mL) under nitrogen. Reaction mixture was stirred at room temperature for 15 minutes prior to dropwise addition of Iodomethane (8.13mL, 130.5mmol), followed by addition of tetrabutylammoniumiodide (0.48g, 1.3mmol) as phase transfer catalyst. Stirring continued at room temperature for a further 90  
15 hours at which time extraction was performed with Ethyl Acetate (3 x 200mL) in the presence of 50% water/50% saturated Sodium Chloride solution (300mL). Combined extracts were washed with water (200mL), 1M Sodium Hydroxide solution (2 x 200mL) and saturated Sodium Chloride solution (200mL) before drying over anhydrous Sodium Sulphate. Evaporation yielded a pale yellow oil  
20 (3.93g, 94.3%) that solidified on standing. TLC analysis indicated only a single component to be present.

### 2-Methoxy- $\beta$ -Estradiol (6)

2-Methoxy- $\beta$ -Estradiol Dibenzy ether (1.41g, 2.9mmol) was dissolved in Ethyl Acetate (30mL) and acidified with 1 M HCL in Diethyl Ether (3mL). 10%  
25 Palladium on Carbon (777mg, 0.73mmol) was added and reaction put on Parr hydrogenator at an initial hydrogen pressure of 65 psi for 48 hours. Catalyst was removed by filtration through a celite pad which was washed with Dichloromethane (3 x 30mL). Evaporation of filtrate gave a pale yellow oil that solidified on standing. TLC analysis indicated two products which were separated  
30 by flash chromatography (Ethyl Acetate : Petroleum Spirit 3:7 v/v and 1:1 v/v) to

yield the product (6) as a cream coloured solid (560mg, 63.8%) and acetate byproduct (7) (230mg, 22.9%) as a white solid.

Further quantities of (6) were obtained from hydrolysis of 2-Methoxy-17 $\beta$ -Acetyloxy-Estradiol (7) (1.39g, 4.0mmol) which was dissolved in THF (25mL) and stirred under nitrogen. An aqueous solution of 0.4M Lithium Hydroxide (22.2mL, 8.9mmol) was added dropwise and stirring continued for 23 hours at room temperature. A small amount of acetate product was present at this time by TLC, with further Lithium Hydroxide (5.0mL, 2.0mmol) solution being added. Reaction mixture continued stirring for a further 1 hour at room temperature prior to acidification to pH 3 with 1M HCl solution. Extraction was performed with 3 x 25mL Dichloromethane with combined extracts being washed with water (25mL) and saturated Sodium Chloride solution (25mL). Drying over anhydrous Sodium Sulphate and evaporation yielded a yellow crystalline solid (1.20g, 98.3%). The product was separated by flash chromatography (Ethyl Acetate : Petroleum Spirit 3:7 v/v and 1:1 v/v) to yield the product(6) as a cream coloured solid (921mg, 75.5%) and acetate starting material (7) (110mg, 7.9%) as a white solid.

#### **2-Methoxy-17 $\beta$ -Estradiol-17-D-triacetylglucopyranosiduronic acid methyl ester (8)**

A mechanically stirred solution of 2-Methoxy- $\beta$ -Estradiol (500mg, 1.65mmol) in toluene (25mL) and Cadmium Carbonate (177mg, 4.95mmol) was refluxed to approximately half volume via a dean stark apparatus ensuring complete dryness of reaction. To this was added dropwise a solution of 1 $\alpha$ -Bromo-2,3,4-tri-O-acetylglucuronate (397mg, 4.95mmol) in anhydrous toluene (25mL) and collection via the dean stark trap continued until volume of reaction mixture was approximately 25mL. Reflux continued for a total of five hours at which time the catalyst was removed by filtration through a celite pad which was thoroughly washed with toluene. Evaporation yielded 1.06g of a buff coloured foam which was purified initially by flash chromatography then preparative HPLC to give 282 mg (27.6%) of product.

**2-Methoxy-17 $\beta$ -Estradiol-17-D-glucopyranosiduronic acid (9)**

To a stirred solution of 9 (245mg, 0.40mmol) in absolute Ethanol 15mL was added 5M Sodium Hydroxide solution (0.5mL). Reaction was stirred at room temperature for 1.5 hours prior to the pH being adjusted to ~3 with 0.2M Sulphuric Acid. Upon evaporation a small sample of the crude mixture (~12mg) was purified by preparative HPLC to yield 7mg of a white washed solid crystalline product (9).

**Example 2: Synthesis of 2-methoxy-3,17 $\beta$ -estradiol- $\beta$ -D-galactopyranosides**

3,17 $\beta$ -Estradiol is initially *bis* protected as the 3-17 $\beta$ -dibenzyl ether by deprotonation with sodium hydride and subsequent reaction with benzyl bromide in DMF in the presence of tetrabutylammonium iodide. After workup the *bis*-protected product is obtained in high yield without the need for lengthy purification. 3-17 $\beta$ -Estradiol dibenzyl ether is brominated at the 2-position, in a THF/acetic acid mixture, with a high degree of regioselectivity (>80%). Recrystallization from ethyl acetate gives pure 2-bromo-3,17 $\beta$ -estradiol dibenzyl ether in good yield, followed by halogen-lithium exchange at low temperature with *t*-butyllithium. Reaction of the lithiated intermediate with MoOPH reagent (a molybdenum pentoxide complex) and subsequent reductive workup yields 2-hydroxy-3,17 $\beta$ -estradiol dibenzyl ether in good yield. Chromatographic purification is required at this point, but is relatively simple given the product impurity profile.

Quantitative methylation is effected by deprotonation of the phenol with potassium carbonate and subsequent alkylation with iodomethane in DMF. Hydrogenolysis of the 2-methoxy dibenzyl protected steroid may be performed under positive hydrogen pressure in the presence of 10% palladium on carbon. The product, 2-methoxy 3-17 $\beta$ -estradiol, is obtained as a white or pale yellow solid. This procedure incorporates a similar synthetic process to that of Cushman et al, 1997, but with improved yields and a more direct and reliable 2-hydroxylation process.

Careful selection of hydrogenolysis conditions, allows selective formation of a number of different products. The 17 $\beta$ -monobenzylether of 2-methoxyestradiol can be produced, as can the 17 $\beta$ -acetate. Depending on subsequent reaction conditions, and the degree of hydroxyl protection required, this can be most advantageous. Protection of the 3-hydroxyl function of 2-methoxyestradiol may be selectively performed by formation of the acetate under mild conditions or further benzyl protection as the 3-monobenzyl ether by standard methods.

Condensation of these compounds via a modified Königs-Knorr method, with 2,3,4,6-tetra-O-acetyl- $\alpha$ -galactopyranosyl bromide in the presence of cadmium carbonate, and subsequent deprotection produces the corresponding 3-( $\beta$ -D) or 17-( $\beta$ -D)-saccharide (Blackwell et al, 1993). The 2,3,4,6-tetra-O-acetyl- $\alpha$ -glucopyranosyl bromide may also be used at this reaction step to give the corresponding 3 or 17 $\beta$ -( $\beta$ -glucopyranosyl) substituted steroids (Paulsen, 1982).

### Example 3

#### 15 Synthesis of various 2-methoxyestradiol (1) analogues and derivatives (See Figure 2)

##### 3-Acetyl 2-methoxyestradiol (2)

2-Methoxyestradiol (105mg, 0.347mmol) was dissolved in anhydrous pyridine (6.0mL) under a nitrogen atmosphere and was cooled to -40°C (acetonitrile/CO<sub>2</sub> bath). Acetic anhydride (3.0mL) was added dropwise over a ten minute period and the reaction left stirring at low temperature for 165 minutes at which time 50mL of 1M HCl was added and the reaction allowed to warm to room temperature. Extraction was performed with ethyl acetate (3x50mL) then the combined organic extracts were sequentially washed with 3M aqueous HCl (50mL), water (50mL) and saturated aqueous NaCl solution (50mL). The organic layers were dried over sodium sulphate then filtered and the solvent evaporated *in vacuo* to give a viscous oil. Purification was performed by flash chromatography (1:1 ethyl acetate/petroleum spirit) to yield the title compound as a white solid (87mg, 71%).

*R*<sub>f</sub> 0.48 [ethyl acetate-petroleum spirit (1:1), silica gel]; <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$  6.87(s,



1H), 6.71(s, 1H), 3.78(s, 3H), 3.71(t, J=8.5Hz, 1H), 2.78-2.73(m, 2H), 2.28(s, 3H), 2.30-1.15(m, 13H), 0.76(s, 3H)

Methyl 3-acetyl-2-methoxyestradiol-17- $\beta$ -D-2',3',4'-tri-O-acetylglucopyranosiduronate (3)

- 5 Freshly dried cadmium carbonate (73.7mg, 0.428mmol) was added to a solution of 3-acetyl-2-methoxyestradiol (2) (36.8mg, 0.107mmol) in anhydrous toluene (40mL) under a nitrogen atmosphere. The suspension was heated at reflux until 20mL of solvent had collected in a Dean-Stark trap. At this time, a solution of methyl 1-bromo-1-deoxy-2,3,4-tri-O-acetyl- $\alpha$ -D-glucopyranuronate (127.4mg, 10 0.321mmol) in toluene (20mL) was added dropwise at approximately the same rate as that of the continuing distillation into the Dean-Stark trap. After 60 minutes of reaction, a further quantity of methyl 1-bromo-1-deoxy-2,3,4-tri-O-acetyl- $\alpha$ -D-glucopyranuronate (84.9mg, 0.214mmol) was added in toluene (10mL) and a further 10mL of solvent distilled. The reaction was then held at reflux for 27 hours 15 before cooling to room temperature. Solids were removed by filtration through a celite pad that was thoroughly washed with toluene. The combined filtrate and washings were sequentially washed with 1M aqueous HCl (30mL), water (30mL), and saturated aqueous NaCl (30mL). The organic layer was dried over sodium sulphate then filtered and evaporated to dryness to yield 133mg of a tan oil.
- 20 Partial purification was performed using flash chromatography (SiO<sub>2</sub>, 0.5:5:94.5 triethylamine/diethylether/dichloromethane) to give the title compound in slightly impure form (67.0mg).

*R<sub>f</sub>* 0.42 [diethylether-dichloromethane (1:19), silica gel].

2-Methoxyestradiol-17- $\beta$ -D-glucopyranosiduronic acid (4)

- 25 Slightly impure glucopyranosiduronate (3) (approx 67.0mg) was dissolved in methanol (15.0mL) and 0.5M NaOH aqueous solution (1.5mL) was added with stirring under a nitrogen atmosphere. Stirring was continued at room temperature for 18 hours at which time the reaction mixture was diluted to approximately 75mL with water and the pH of the reaction mixture was adjusted to pH 8.0 with 0.5M

aqueous HCl. The aqueous solution was washed with diethyl ether (2x10mL), prior to further pH adjustment to pH 2.5. The aqueous solution was washed with dichloromethane (2x10mL) and extraction performed with *t*-butyl methyl ether (5x10mL). The combined *t*-butyl methyl ether layers were dried over sodium sulphate then filtered and the solvent evaporated *in vacuo* to give the title compound as a white/cream crystalline solid (24.8mg, 48.5% over the two step process).

mp 181-182°C (dec); <sup>1</sup>H NMR (CD<sub>3</sub>OD): δ 6.79(s, 1H), 6.47(s, 1H), 4.41(d, J=7.6Hz, 1H) 3.79(s, 3H), 3.75(t, J=8.4Hz, 1H), 3.51(t, J=9.4Hz, 1H), 3.36(t, J=9.2Hz, 1H), 3.31-3.29(m, 1H), 3.22(t, J=8.4Hz, 1H), 2.78-2.62(m, 2H), 2.33-2.24(m, 1H), 2.20-1.94(m, 3H), 1.73-1.63(m, 2H), 1.50-1.16(m, 6H), 0.87(s, 3H); K[partition coefficient] (1-octanol/water) = 0.372.

### 3.17-Diacetyl-2-methoxyestradiol (5)

2-Methoxyestradiol (127.5mg, 0.426mmol) was dissolved in anhydrous pyridine (6.0mL) under a nitrogen atmosphere and cooled to 0°C. Acetic anhydride (3.0mL) was added dropwise and the reaction allowed to warm to room temperature. After overnight stirring, the reaction mixture was cooled to 0°C at which time 50mL of 1M aqueous HCl was added and the reaction allowed to warm to room temperature. Extraction was performed with ethyl acetate (3x50mL) and the combined combined organic extracts were sequentially washed with 3M aqueous HCl (50mL), water (50mL) and saturated aqueous NaCl (50mL). The organic layers were dried over sodium sulphate then filtered and the solvent evaporated *in vacuo* to give a white solid (154mg). The product was homogeneous when analysed by TLC therefore purification was deemed unnecessary, however an analytical sample was purified on a silica gel column using 1:5 ethyl acetate/petroleum spirit as eluant.

*R<sub>f</sub>* 0.88[ethyl acetate-petroleum spirit (1:1), silica gel]; <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 6.86(s, 1H), 6.71(s, 1H), 4.67(t, J=8.3Hz, 1H), 3.78(s, 3H), 2.77-2.74(m, 2H), 2.28(s, 3H), 2.04(s, 3H), 2.26-1.23(m, 13H), 0.81(s, 3H)

17-Acetyl-2-methoxyestradiol (6)

Activated zinc (4x1g portions) was added to a stirred solution of 3,17-diacetyl-2-methoxyestradiol (5) (154mg) in methanol (25mL) at time points of 0, 64, 94 and 118 hours. After this time, solids were removed by filtration through a celite pad  
5 that was then thoroughly washed with methanol and dichloromethane. The combined organic filtrates and washings were washed with water (10mL) and saturated aqueous NaCl solution (10mL) prior to drying over sodium sulphate and filtration. Evaporation of the organic solvents *in vacuo* yielded 103.5 mg of a white crystalline solid. (103.5mg, 71.3% over the two step process).

10  $R_f$  0.70[ethyl acetate-petroleum spirit-acetic acid (20:78:2), silica gel];  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$ 6.86(s, 1H), 6.71(s, 1H), 4.67(t,  $J=8.3\text{Hz}$ , 1H), 3.78(s, 3H), 2.77-2.74(m, 2H), 2.04(s, 3H), 2.26-1.23(m, 13H), 0.81(s, 3H).

3,17-Diacetyl-6-oxo-2-methoxyestradiol (7)

Chromium trioxide (79.8mg, 0.798mmol) was dissolved with stirring over a 90  
15 minute period in 2.0mL of 90% (v/v) aqueous acetic acid. This oxidizing agent was added dropwise at 10°C to a stirred solution of 3,17-diacetyl-2-methoxyestradiol (5) (72.5mg, 0.188mmol). The mixture was stirred at 10°C for 30 minutes at which time the reaction was poured onto an ice/water mixture (30mL) which was extracted with ethyl acetate (3x15mL). The combined extracts were washed with  
20 water (20mL), saturated aqueous  $\text{NaHCO}_3$  (20mL), water (20mL), and saturated aqueous NaCl (20mL) prior to drying over sodium sulphate and filtration. Evaporation of the organic solvent yielded a solid yellow residue. This residue was purified by flash chromatography on silica gel using a 1:3 ethyl acetate/petroleum spirit mixture as eluant to give a white solid (34mg, 45.3%).

25  $R_f$  0.36[ethyl acetate-petroleum spirit (1:3), silica gel];  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$ 7.73(s, 1H), 6.92(s, 1H), 4.71(t,  $J=8.5\text{Hz}$ , 1H), 3.90(s, 3H), 2.69(dd,  $J=16.9$ , 3.4Hz, 1H), 2.53(dt,  $J=16.9$ , 3.4Hz, 1H), 2.31(s, 3H), 2.06(s, 3H), 2.40-1.34(m, 13H), 0.83(s, 3H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$ 195.81, 171.10, 168.92, 155.40, 146.95, 138.42, 126.06,

121.79, 108.36, 82.09, 55.97, 49.74, 43.49, 43.26, 42.63, 39.57, 36.38, 27.36, 25.35, 22.92, 21.12, 20.52, 11.84.

#### 6-Oxo-2-methoxyestradiol (8)

Anhydrous potassium carbonate (20mg, 0.145mmol) was added to a stirred  
5 solution of 3,17-diacetyl-6-oxo-2-methoxyestradiol (7) (34mg, 0.085mmol) in  
8.0mL of methanol under a nitrogen atmosphere. Water (3.0mL) was added and  
reaction stirred at room temperature for 16 hours. At this time, the pH of the  
reaction was adjusted to pH 4 with dropwise addition of 1M HCl aqueous solution.  
Extraction was performed with ethyl acetate (3x10mL) then the combined layers  
10 were washed with water (10mL) and saturated aqueous NaCl solution (10mL)  
prior to drying over sodium sulphate and filtration. Evaporation of the solvent *in vacuo*  
yielded a cream coloured solid which was purified by flash chromatography  
on silica gel (3:2 ethyl acetate: petroleum spirit) to give 26.4mg (98.3%) of the  
target compound as a white crystalline solid.

15 mp 189-190°C (dec);  $R_f$  0.34[ethyl acetate-petroleum spirit (3:2), silica gel];  $^1\text{H}$   
NMR ( $\text{CD}_3\text{OD}$ ):  $\delta$ 7.36(s, 1H), 6.91(s, 1H), 3.92(s, 3H), 3.66(t,  $J=8.5\text{Hz}$ , 1H),  
2.52(dd,  $J=16.8, 3.5\text{Hz}$ , 1H), 2.43-1.26(m, 12H), 0.76(s, 3H);

#### 3-Acetyl 6-oxo-2-methoxyestradiol (9)

6-Oxo-2-methoxyestradiol (8) was dissolved in anhydrous pyridine (4.0mL) with  
20 stirring under a nitrogen atmosphere and the solution cooled to -40°C  
(acetonitrile/ $\text{CO}_2$  bath). Acetic anhydride (2.0mL) was added dropwise over a ten  
minute period at this temperature, and the reaction was allowed to stir at -40°C for  
165 minutes prior to quenching with water (10mL). Extraction was performed with  
ethyl acetate (3x20mL) and the combined organic layers were washed with 3M  
25 aqueous HCl (2x20mL), saturated aqueous NaCl (20mL) and dried over sodium  
sulphate then filtered prior to evaporation of the solvent *in vacuo* to yield a pale  
yellow oil (30.2mg). Purification of the title compound was performed by short  
column chromatography on silica gel (1:3 ethyl acetate/petroleum spirit) to give a

white solid (14.2mg, 47.5%).

$^1\text{H}$  NMR ( $\text{C}_6\text{D}_6$ ):  $\delta$ 8.31(s, 1H), 6.54(s, 1H), 3.30(s, 3H), 3.37(t,  $J=8.6\text{Hz}$ , 1H), 3.30(s, 3H), 2.60(dd,  $J=16.8, 3.6\text{Hz}$ , 1H), 2.01-0.63(m, 12H), 0.57(s, 3H);

3,17-Diacetyl-2-methoxyestradiol-6-hydrazone (10)

5 Sodium acetate (144mg, 1.755mmol) was suspended in a solution of 3,17-diacetyl-6-oxo-2-methoxyestradiol (7) (35.5mg, 0.089mmol) in anhydrous methanol (25.0mL) under a nitrogen atmosphere. Hydrazine hydrate (6 drops) was added and the reaction stirred at room temperature for 2 hours. The reaction mixture was then heated to reflux for a further 2 hours and then glacial acetic acid  
10 (10 drops) was added. After this, the reaction continued at reflux for a further 4.5 hours then glacial acetic acid (5 drops) was added. The reaction was continued at reflux for a further 1.5 hours after which time a further 8 drops of glacial acetic acid and 5 drops of hydrazine hydrate were added and reflux continued for 3 hours. Upon cooling to room temperature, the solvents were evaporated to give a  
15 bright yellow semi-solid. This residue was dissolved in 20mL of water and extraction performed with dichloromethane (3x50mL). The combined organic layers were then washed with water (20mL) and dried over sodium sulphate then filtered. Evaporation of the solvent *in vacuo* gave the target hydrazone product.

$R_f$  0.24[ethyl acetate-petroleum spirit (1:1), silica gel];  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$ 7.86(s, 20 1H), 6.81(s, 1H), 4.69(t,  $J=8.3\text{Hz}$ , 1H), 3.94(s, 3H), 3.37(dd,  $J=17.6, 3.9\text{Hz}$ , 1H), 2.06(s, 3H), 2.04(s, 3H), 2.38-1.21(m, 12H), 0.83(s, 3H); ESIMS-  $M+1$   $m/z$  = 415.1.

2-Methoxyestradiol-6-hydrazone (11)

A small sample (ca. 5mg) of 3,17-diacetyl-2-methoxyestradiol-6-hydrazone (10) was suspended in anhydrous methanol (75mL) in the presence of a large excess  
25 of anhydrous potassium carbonate. The reaction mixture was stirred overnight at room temperature and the pH adjusted to pH 5 with a combination of 1M aqueous  $\text{NH}_4\text{Cl}$  and 2M aqueous HCl. The residual methanol was evaporated *in vacuo* and the aqueous residue extracted with dichloromethane (3x50mL). The combined

organic phases were washed with water and dried over sodium sulphate then filtered. Evaporation of the solvent *in vacuo* yielded a yellow solid that was recrystallised from chloroform.

$R_f$  0.30[ethyl acetate-petroleum spirit (4:1), silica gel].

#### 5 Example 4

##### Susceptibility of 2-methoxy-estradiol-17 $\beta$ -D-glucuronide to metabolism by glucuronidase.

2-MEG was incubated with purified E. Coli-derived  $\beta$ -glucuronidase for 16 hours (rat renal homogenate for 5.5 hours) in Tyrodes buffer. At the end of these incubation periods, extraction was carried out by using 100  $\mu$ l of reaction mixture with 200  $\mu$ l of acetonitrile (ACN) followed by the addition of 5  $\mu$ l 2M HCL, vortexing and centrifugation at 1500g (ambient temperature). Samples of enzyme incubates or quality control incubates were then chilled at -20°C for at least 10 min, prior to removal of 120  $\mu$ l of the upper (ACN) phase which is added to 120  $\mu$ l distilled H<sub>2</sub>O. One hundred  $\mu$ l of this extract was injected into a Hewlett Packard Series 1050 HPLC fitted with a C18 analytical column (Hypersil ODS 3 $\mu$ M, 4.6 x 150mm) guarded by a pre column (Alltech Hypersil ODS 3  $\mu$ M; 4.6 x 7.6 mm) and metabolites were detected with a Hewlett Packard 1046A fluorescence detector (excitation 270 nm, emission, 320 nm). Metabolites were separated in a mobile phase of ACN buffer of 40:60 (buffer comprised 20 mM NaH<sub>2</sub> PO<sub>4</sub>, 0.02% orthophosphoric acid, pH 3.1 - 3.2) at a flow rate of 0.7 ml/min. for the first 4 min with a linear gradient to a mobile phase of ACN: buffer (76:24) at 4.5 min. which was maintained for the remainder of the separation (10 min).

Quantitative recovery of quality control standards was achieved. 2-MEG eluted with a retention time of 4.35 min and 2-MEO of 9.8 min. The fluorescence of 2-MEO was approximately 5 fold that of 2-MEG (Figure 3). It is evident that appreciable conversion of 2-MEG to 2-MEO occurs during incubation with either E.Coli  $\beta$ -glucuronidase (~20% conversion) and rat renal homogenates (~6%)

during incubation periods of 16 hours and 5.5 hours, respectively.

### Example 5

#### Regulation of airway mesenchymal cell number and DNA synthesis by 2-methoxyestradiol and 2-methoxyestradiol-17 $\beta$ -D-glucuronide

5 Human airway fibroblasts were obtained from volunteers in a follow-up of the Melbourne Epidemiological Survey of Childhood Asthma (MESCA) (Oswald *et al.*, 1994) initially recruited at age 7 in 1965 and now 42 years old. Volunteers underwent bronchial biopsy which provided tissue samples of epithelium and mucosa of approximately 2 mm<sup>3</sup>. These specimens were dissected to remove the  
10 epithelium and placed in plastic culture plates. Fibroblasts grew by explant culture over a six week period when maintained according to previously described methods (Zhang *et al.*, 1996). Cells were seeded at subconfluent densities onto six cell plates and after 72 hours were rendered quiescent by serum deprivation. Twenty four hours after serum removal, cells were exposed to thrombin (0.3 U/ml)  
15 together with a serum-free medium supplement containing insulin, transferrin and selenium. Cells were harvested by incubation with Trypsin (0.5% w/v) for 10 min. or until a single cell suspension was produced. Cell numbers were then determined using a haemocytometer.

Airway smooth muscle cells maintained in culture for 72 hours in the presence of  
20 5% FCS and 2-MEO (10 micro M) underwent a readily discernible shape change (Figure 4) that was not detected for cells incubated for the same period and at the same concentration of MEG, suggesting that MEG lacked this active of the parent molecule.

Smooth muscle cells were obtained from airways resected from lung transplant  
25 recipients and cultured as previously described in detail (Fernandes *et al.*, 1999). Cell numbers were determined in experiments of the same design as those described for human airway fibroblasts with the additional stimulus of 1% (v/v) fetal calf serum (FCS) being evaluated. In addition, [<sup>3</sup>H]-thymidine incorporation was determined between 24 and 28 hours after the addition of thrombin to

determine DNA synthesis (Fernandes *et al.*, 1999).

In human airway fibroblasts thrombin-stimulated increases in cell number were reduced to  $12 \pm 8\%$  of the control response by 2-MEO, but 2-MEG had no significant effect (Table 1). In human airway smooth muscle responses both  
5 thrombin and FCS were reduced to a greater extent by 2-MEO than by 2-MEG. In addition, thrombin-stimulated DNA synthesis was decreased by 2-MEO, but unaffected by 2-MEG (Table 1).

A549 cells were maintained in DMEM containing 5% fetal calf serum and undergoing asynchronous proliferation. DNA synthesis was determined by the  
10 addition of [3H]-thymidine 24 hours after the addition of one of 2-methoxyestradiol, 6-oxo-2-methoxyestradiol and 2-methoxyestradiol-17 $\beta$ -D-glucuronide. Both 2-methoxyestradiol and 6-oxo-2-methoxyestradiol at 10 micromolar reduce DNA synthesis significantly whereas 2-methoxyestradiol-17 $\beta$ -D-glucuronide was ineffective (Figure 5).

15 These data indicate that 2-MEG is either less active or inactive by comparison with the parent molecule, 2-MEO.

### Example 6

#### Rat uterine cytosol estrogen receptor binding

Rat uterine cytosol is a standard preparation of estrogen receptor (Markavevich  
20 *et al.*, 19xx). Uteri from four Sprague Dawley rats were pooled, homogenised in 10 ml/g wet weight of buffer (pH 7.4, 0.002 M dithiothreitol, 0.25 M Sucrose) by 3 x 10s bursts in an ultra Turrax homogeniser. The resulting homogenate was subjected to centrifugation at 1000 x g for 5 min at 4°C to remove nuclei and cellular debris. The supernatant was subjected to centrifugation at 35,000 x g for  
25 30 min. at 4°C in a Beckman JA centrifuge. The supernatant was used in binding assays that were conducted in the same buffer. The displacement of 0.2 nM [<sup>3</sup>H]-estradiol (150 Ci/mmol) was examined by incubating 200  $\mu$ l of rat uterine cytosol with 50  $\mu$ L of radioactive estradiol and 50  $\mu$ L of buffer containing concentrations of



estradiol (non-radiolabelled) in the range 0.003 nM to 10 nM; 2-MEO 0.003 to 10  $\mu$ M; 2-MEG 0.003 to 10  $\mu$ M. Total binding was determined in incubations in which buffer was added in place of displacing agent and 10  $\mu$ M estradiol was used to define non-specific binding.

- 5 Each of the displacers produced displacement but there was an incomplete response at 10  $\mu$ M 2-MEO that  $\log IC_{50}$  values indicate that 2-MEG has only 1/50 the affinity of 2-MEO for estrogen receptors (Figure 6). It is evident that 2-MEO has significant affinity for estrogen receptors over the concentration range that reduces mesenchymal cell proliferation with a  $IC_{50}$  of approximately 100 nM, even  
10 though its affinity is considerably less than that of its precursor estradiol ( $IC_{50}$  3 nM).

### Example 7

#### Relative solubilities of 2-MEO and MEG

- 15 The octanol:water partition co-efficient is a measure of the relative aqueous and lipid solubility of a compound. For 2-MEO this was determined by fluorescence spectroscopy at  $Ex = 250nm$ ,  $Em = 325nm$  on the aqueous solution after extensive mixing to ensure that an equilibrium had been obtained. The measured octanol/water partition coefficient for 2methoxyestradiol is 9.05 ( $\log P = 0.957$ ), and  
20 for MEG it is 0.372 ( $\log P = -0.429$ ) using fluorescence spectroscopy at  $Ex = 240nm$ ,  $Em = 352nm$ . Thus, the aqueous solubility of the glucuronide is approximately 25 times that of 2-MEO itself.

**Table 1.** The effects of 2-MEO and MEG on proliferation of human airway fibroblastst or smooth muscle cells measured by DNA synthesis or by increases in cell number.

### Human Airway Fibroblasts

5

Cell Number (% control)

| Stimulus           | 2-MEO (10 $\mu$ M) | 2-MEG (10 $\mu$ M) |
|--------------------|--------------------|--------------------|
| Thrombin (0.3U/ml) | 12 $\pm$ 8, n=7    | 123 $\pm$ 33, n=10 |

### Human Airway Smooth Muscle

Cell Number (% control)

| Stimulus            | 2-MEO (10 $\mu$ M) | 2-MEG (10 $\mu$ M) |
|---------------------|--------------------|--------------------|
| Thrombin (0.3 U/ml) | 14 $\pm$ 12, n=10  | 29 $\pm$ 8, n=12   |
| FCS (1%, v/v)       | 25 $\pm$ 9, n=5    | 47 $\pm$ 16, n=5   |

10

### Human Airway Smooth Muscle

DNA Synthesis (% control)

| Stimulus            | 2-MEO (10 $\mu$ M) | 2-MEG (10 $\mu$ M) |
|---------------------|--------------------|--------------------|
| Thrombin (0.3 U/ml) | 24 $\pm$ 8, n=10   | 91 $\pm$ 16, n=4   |

## References

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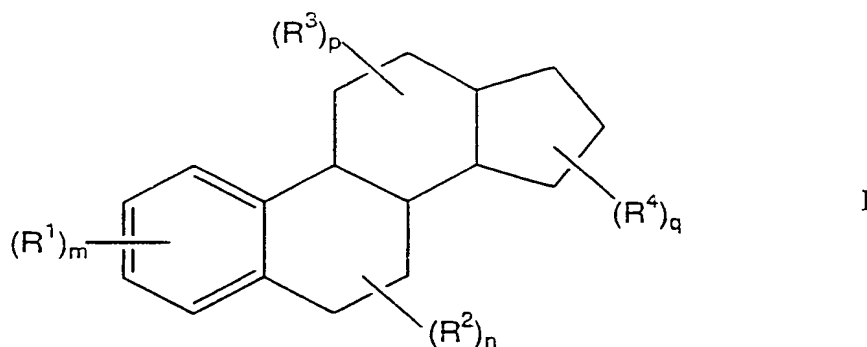
Finally, it is to be understood that various alterations, modifications and/or additions may be made without departing from the spirit of the present invention as outlined herein

- 5 It will be understood that the invention disclosed and defined in this specification extends to all alternative combinations of two or more of the individual features mentioned or evident from the text or drawings. All of these different combinations constitute various alternative aspects of the invention.

- 10 It will also be understood that the term "comprises" (or its grammatical variants) as used in this specification is equivalent to the term "includes" and should not be taken as excluding the presence of other elements or features.

## CLAIMS

1. A conjugated prodrug of an estradiol compound conjugated to a biological activity modifying agent.
2. A conjugated prodrug according to claim 1 wherein the biological activity modifying agent also functions as a masking and/or targetting agent.
3. A conjugated prodrug according to claim 1 wherein the biological activity modifying agent includes a carbohydrate, protein or peptide moiety.
4. A conjugated prodrug according to claim 3 wherein the carbohydrate moiety is a sugar moiety.
5. A conjugated prodrug according to claim 4, wherein the sugar moiety is present, or is present at increased levels, in the condition to be treated.
6. A conjugated prodrug according to claim 5, wherein the sugar moiety is a glucuronide or galactoside.
7. A conjugated prodrug according to claim 1, wherein the estradiol compound is 2-methoxyestradiol or a functionally active analogue or derivative thereof.
8. A conjugated prodrug according to claim 3 wherein the biological activity modifying agent is a peptide moiety.
9. A conjugated prodrug according to claim 8 wherein the peptide moiety is a tri- or higher peptide residue.
10. A compound having the formula I



wherein m, n, p and q are each integers of 0, 1, 2 or 3,

each  $R^1$ ,  $R^2$ ,  $R^3$  and  $R^4$  is substituent independently selected from one or more of hydrogen, hydroxy, halo, optionally substituted  $C_{1-6}$  alkyl,  $C_{1-6}$  alkenyl,  $C_{1-6}$  alkynyl,  $C_{3-8}$  cycloalkyl,  $C_{1-6}$  alkanoyl,  $C_{1-6}$  alkoxy, aryl, amino, acyl,  $C_{1-6}$  acyloxy aminoalkyl, imino,  $C_{1-6}$  iminoalkyl,  $C_1$ - $C_3$  imino cycloalkyl, keto, aldehyde, hydrazono, oximino, hydroximino,  $C_{1-6}$  alkoximino,  $C_{3-8}$  cycloalkoximino or carboxyl groups, or an O-Protecting group, or a carbohydrate, peptide or protein residue, or derivative thereof or a pharmaceutically acceptable salt or in vivo hydrolysable ester, amide carbonate, or carbamate thereof:

with the proviso that at least one of  $R^1$  to  $R^4$  is present.

11. A compound according to claim 10, wherein each of  $R^1$  to  $R^4$  is selected from hydrogen hydroxy, halo, optionally substituted  $C_{1-6}$  alkyl,  $C_{1-6}$  alkoxy, imino,  $C_{1-6}$  iminoalkyl,  $C_{3-8}$  iminocycloalkyl, keto, aldehyde, hydroximino, alkoximino, hydrazono, carbamate, an O-Protecting group, or a sugar protein or peptide residue.

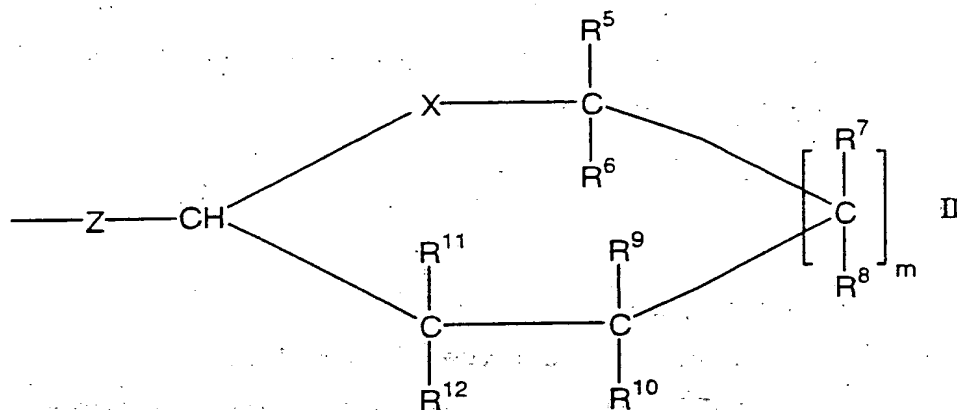
12. A compound according to claim 11, wherein each of  $R^1$  to  $R^4$  is selected from hydroxy,  $C_{1-6}$  alkoxy, imino  $C_{3-8}$  iminocycloalkyl, keto, hydrazono, carbamate, or a sugar, protein, or peptide residue.

13. A compound according to claim 10:  
 wherein  $R^1$  is one or more of hydroxy, alkoxy, acyloxy or a carbohydrate, protein or peptide residue;  
 $R^3$  includes an alkyl group; and  
 $R^4$  is one or more of hydroxy, alkoxy, an O-Protecting group or a carbohydrate, protein or peptide residue.

14. A compound according to claim 13:  
wherein  $R^1$  includes a methoxy group.

15. A compound according to claim 14 wherein:  
 $R^3$  includes one or more of H, a keto, or a hydrazono group.

5 16. A compound according to claim 11 wherein:  
 $R^1$ ,  $R^2$  or  $R^4$  includes a sugar residue having the formula II:



wherein Z is a spacer or bond;

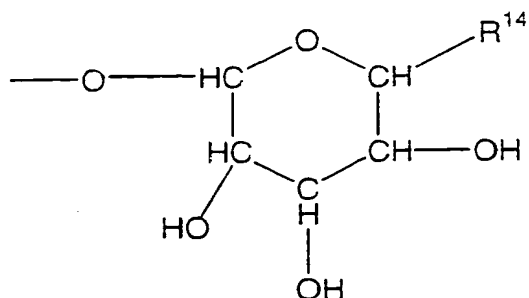
$m$  is 0 or 1; and

10 each of  $R^5$  to  $R^{12}$  are independently selected from H, OH, amino, halo,  $COOR^{13}$ ,  $CH_2OR^{13}$ ,  $CHOH-COOR^{13}$ ,  $PO_3H_2$ ,  $CH_2-PO_3H_2$  or  $CHOH-PO_3H_2$  24  $CH_2COOR^{13}$ ,  $OCOR^{13}$ ;

wherein  $R^{13}$  is H or optionally substituted  $C_1$  to  $C_6$  alkyl,  $C_{1-6}$  alkenyl,  $C_{3-8}$  cycloalkyl,  $C_{1-6}$  alkanoyl,  $C_{1-6}$  alkoxy, aryl, amino, acyl,  $C_{1-6}$  acyloxy aminoalkyl,

15 imino,  $C_{1-6}$  iminoalkyl, keto, hydrazono, or carboxyl groups.

17. A compound according to claim 16 wherein the sugar residue has the formula:



wherein  $R^{14}$  is  $-\text{CH}_2\text{OH}$  or  $-\text{COOH}$ .

18. A conjugated prodrug according to claim 10 wherein the peptide residue has the formula III:



wherein Z is a spacer or bond

m, n and p are each integers of 0, 1 or 2

ABCDGHIJK, which may be the same or different, are each amino acids.

19. A conjugated prodrug according to claim 18 wherein the peptide is,  
10 or includes the sequence -Ala-Phe-Lys or -Val-Leu-Lys.

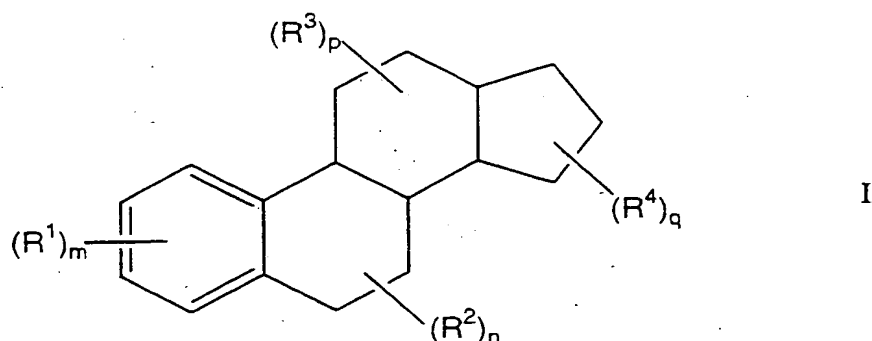
20. A compound according to claim 10, selected from:

- 3-Acetyl 2-methoxyestradiol  
 3-Acetyl 6-oxo-2-methoxyestradiol  
 3,17-Diacetyl-2-methoxyestradiol  
 15 17-Acetyl-2-methoxyestradiol  
 3,17-Diacetyl-6-oxo-2-methoxyestradiol  
 3,17-Diacetyl-2-methoxyestradiol-6-hydrazone  
 2-Methoxyestradiol-6-hydrazone  
 2-Methoxy estradiol-17 $\beta$ -monodienyl ether  
 20 2-Methoxy estradiol-17 $\beta$ -acetate  
 2-Methoxy-17 $\beta$ -Estradiol-17-D-triacetylglucopyranosiduronic acid methyl ester



2-Methoxy-17 $\beta$ -Estradiol-17-D-glucopyranosiduronic acid  
 2,3,17 $\beta$ -estradiol-17- $\beta$ -D-galactopyranoside-2-methyl ether, or  
 2,3,17 $\beta$ -estradiol-3- $\beta$ -D-galactopyranoside-2-methyl ether.

21. A conjugated prodrug of the formula I:



5

wherein m, n, p and q are each integers of 0, 1, 2 or 3,

each R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup> and R<sup>4</sup> is substituent independently selected from one or more of hydrogen, hydroxy, halo, optionally substituted C<sub>1-6</sub> alkyl, C<sub>1-6</sub> alkenyl, C<sub>1-6</sub> alkynyl, C<sub>3-8</sub> cycloalkyl, C<sub>1-6</sub> alkanoyl, C<sub>1-6</sub> alkoxy, aryl, amino, acyl, C<sub>1-6</sub> acyloxy aminoalkyl, imino, C<sub>1-6</sub> iminoalkyl, C<sub>1-C3</sub> imino cycloalkyl, keto, aldehyde, hydrazono, oximino, hydroximino, C<sub>1-6</sub> alkoximino; C<sub>3-8</sub> cycloalkoximino or carboxyl groups, or an O-Protecting group, or a carbohydrate, peptide or protein residue, or derivative thereof or a pharmaceutically acceptable salt or in vivo hydrolysable ester, amide carbonate, or carbamate thereof:

10 with the proviso that at least one of R<sup>1</sup> to R<sup>4</sup> is present and includes a carbohydrate, protein or peptide residue.

15

22. A conjugated prodrug according to claim 21:

wherein

R<sup>1</sup> is one or more of hydroxy, alkoxy, acyloxy or a carbohydrate, peptide or protein residue;

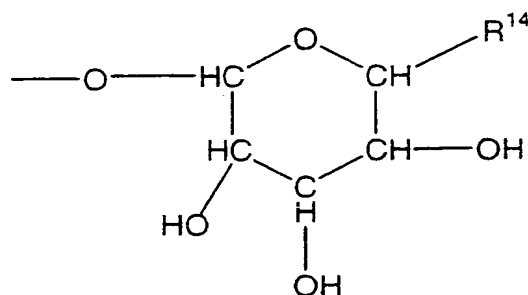
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R<sup>3</sup> includes an alkyl group; and

R<sup>4</sup> is one or more of hydroxy, alkoxy, O-Protecting groups or a carbohydrate, protein or peptide residue.

23. A conjugated prodrug according to claim 22 wherein R<sup>1</sup> includes a methoxy group.

24. A conjugated prodrug according to claim 21 wherein the sugar residue has the formula:



5

wherein R<sup>14</sup> is -CH<sub>2</sub>OH or -COOH.

25. A conjugated prodrug according to claim 21 wherein the peptide residue has the formula III:



10

wherein Z is a spacer or bond

m, n and p are each integers of 0, 1, 2

ABCDGHIJK, which may be the same or different, are each amino acids.

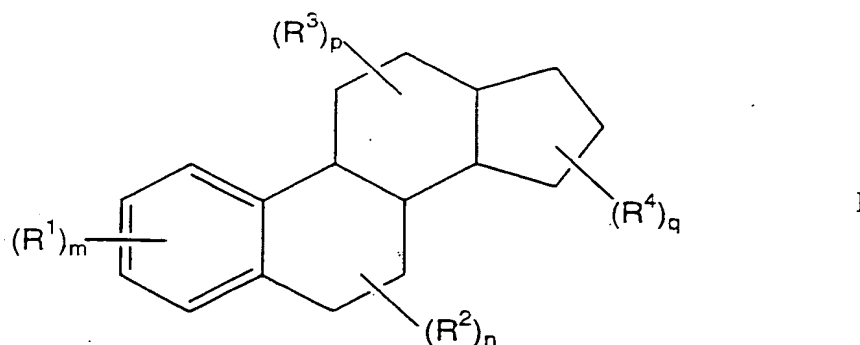
26. A pharmaceutical composition including:  
a conjugated prodrug of an estradiol compound conjugated to a biological  
15 activity modifying agent; and  
a pharmaceutically acceptable carrier, diluent or excipient therefor.

27. A pharmaceutical composition according to claim 26 wherein the estradiol compound is 2-methoxyestradiol or a functionally active analogue or derivative thereof.

20

28. A pharmaceutical composition according to claim 26 wherein the

conjugated prodrug has the formula:



wherein m, n, p and q are each integers of 0, 1, 2 or 3,

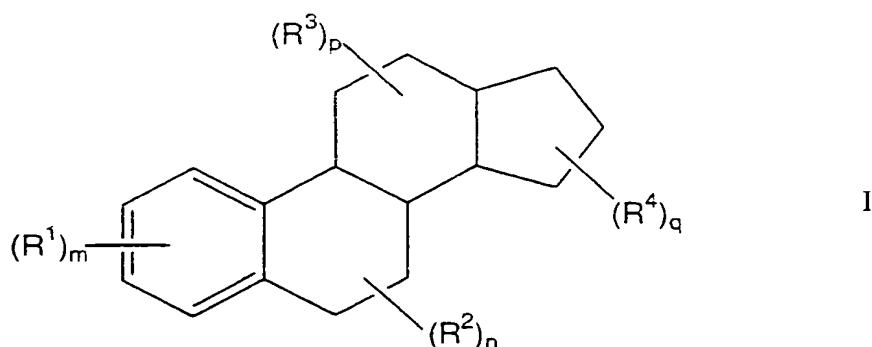
each R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup> and R<sup>4</sup> is substituent independently selected from one or  
 5 more of hydrogen, hydroxy, halo, optionally substituted C<sub>1-6</sub> alkyl, C<sub>1-6</sub> alkenyl, C<sub>1-6</sub>  
 alkynyl, C<sub>3-8</sub> cycloalkyl, C<sub>1-6</sub> alkanoyl, C<sub>1-6</sub> alkoxy, aryl, amino, acyl, C<sub>1-6</sub> acyloxy  
 aminoalkyl, imino, C<sub>1-6</sub> iminoalkyl, C<sub>1-3</sub> imino cycloalkyl, keto, aldehyde,  
 hydrazono, oximino, hydroximino, C<sub>1-6</sub> alkoximino, C<sub>3-8</sub> cycloalkoximino or carboxyl  
 groups, or an O-Protecting group, or a carbohydrate, peptide or protein residue, or  
 10 derivative thereof or a pharmaceutically acceptable salt or in vivo hydrolysable  
 ester, amide carbonate, or carbamate thereof:

with the proviso that at least one of R<sup>1</sup> to R<sup>4</sup> is present and includes a  
 carbohydrate protein or peptide residue.

29. A method of prophylaxis or treatment of a condition associated with  
 15 angiogenesis or accelerated or increased amounts of cell division, or  
 inflammation, said method including administering to a patient, in need of such  
 prophylaxis or treatment, an effective amount of a conjugated prodrug of an  
 estradiol compound conjugated to a biological activity modifying agent.

30. A method according to claim 29 wherein the estradiol compound is  
 20 2-methoxyestradiol or a functionally active analogue or derivative thereof.

31. A method according to claim 30 wherein the conjugated prodrug has  
 the formula:



wherein m, n, p and q are each integers of 0, 1, 2 or 3,

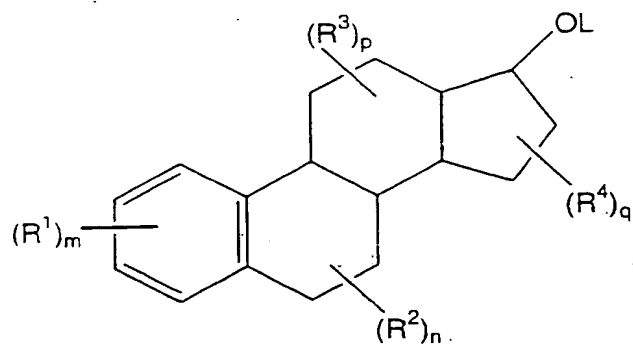
each  $R^1$ ,  $R^2$ ,  $R^3$  and  $R^4$  is substituent independently selected from one or more of hydrogen, hydroxy, halo, optionally substituted  $C_{1-6}$  alkyl,  $C_{1-6}$  alkenyl,  $C_{1-6}$  alkynyl,  $C_{3-8}$  cycloalkyl,  $C_{1-6}$  alkanoyl,  $C_{1-6}$  alkoxy, aryl, amino, acyl,  $C_{1-6}$  acyloxy aminoalkyl, imino,  $C_{1-6}$  iminoalkyl,  $C_1$ - $C_3$  imino cycloalkyl, keto, aldehyde, hydrazono, oximino, hydroximino,  $C_{1-6}$  alkoximino,  $C_{3-8}$  cycloalkoximino or carboxyl groups, or an O-Protecting group, or a carbohydrate, peptide or protein residue, or derivative thereof or a pharmaceutically acceptable salt or in vivo hydrolysable ester, amide carbonate, or carbamate thereof:

with the proviso that at least one of  $R^1$  to  $R^4$  is present and includes a carbohydrate, peptide or protein residue.

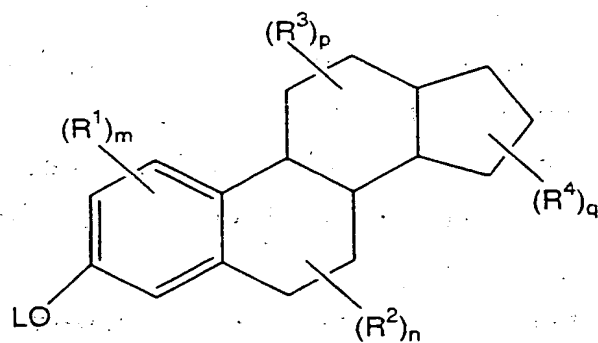
32. A method of preparing a compound of formula I which method includes:

- 15 (a) reacting a compound of formula IV, V or VI with a compound of formula VII or VIII:

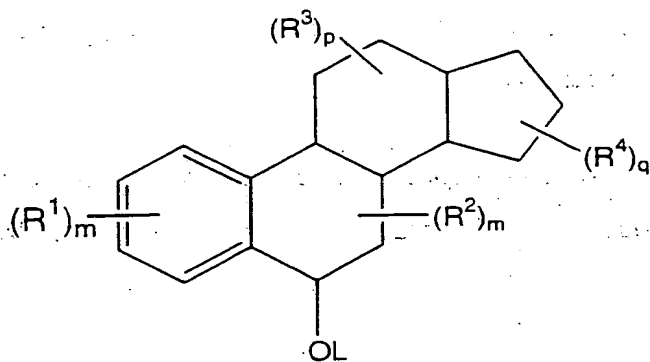
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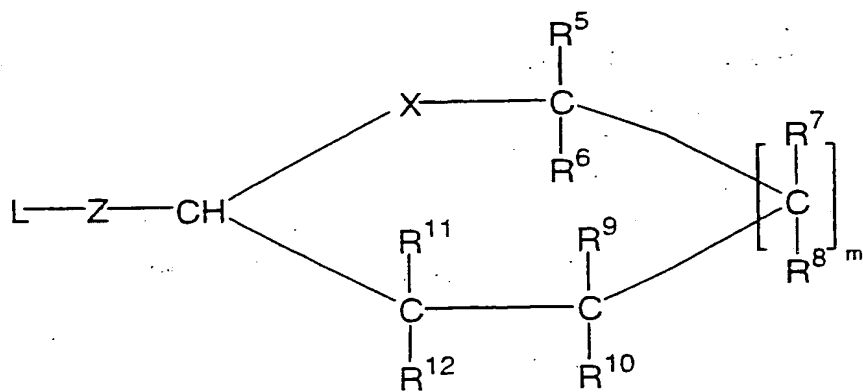
IV



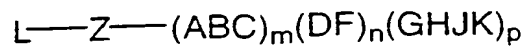
V



VI



VII



VIII

wherein  $R^1$ ,  $R^2$ ,  $R^3$ ,  $R^4$ , ABCDEFGHJK and m, n, p and q are as defined in claim 10 and 18 except that in formula IV,

q is 0, 1 or 2;

in Formula V, m is 0, 1 or 2 and

5 in Formula VI, n is 0, 1 or 2

$R^5$  to  $R^{12}$  are as defined in claim 16,

L is a leaving group,

Z is a spacer or bond;

wherein any functional group is protected, if necessary; and

10 (b) removing any protecting groups;

(c) optionally, before or after said reaction converting any compound of formula III, V or VI into another compound of formula IV, V or VI;

(d) optionally forming a pharmaceutically acceptable salt or in vivo hydrolysable ester, amide, carbonate or carbamate.

15 33. A method according to claim 32, wherein the reaction is conducted in the presence of a catalyst.

34. A conjugated prodrug, or compound of formula I, substantially as hereinbefore described with reference to any one of the examples.

20 The University of Melbourne  
By its Registered Patent Attorneys  
**Freehills Carter Smith Beadle**

13 October 2000



FIGURE 2

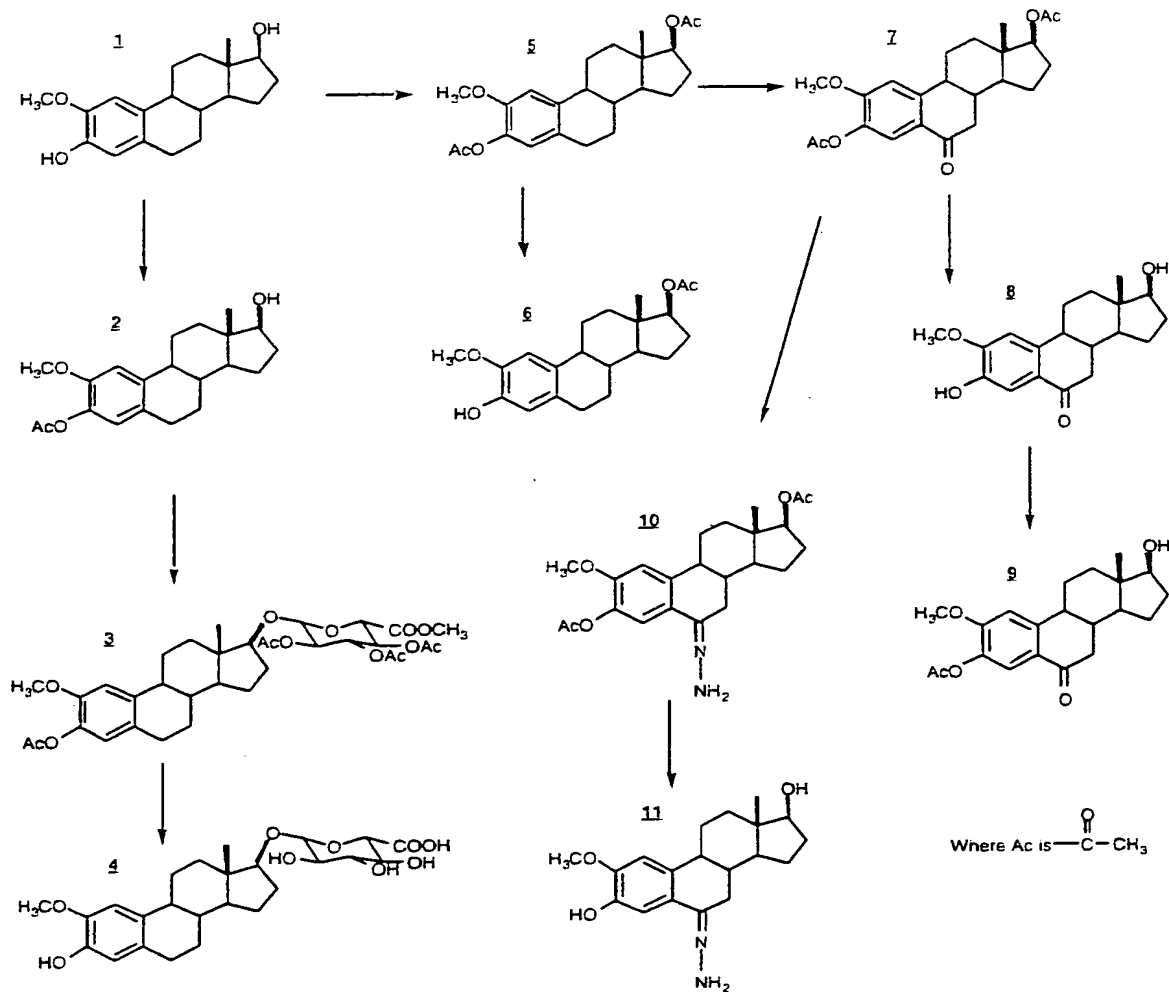




Figure 3  
Arbitrary Fluorescence Units

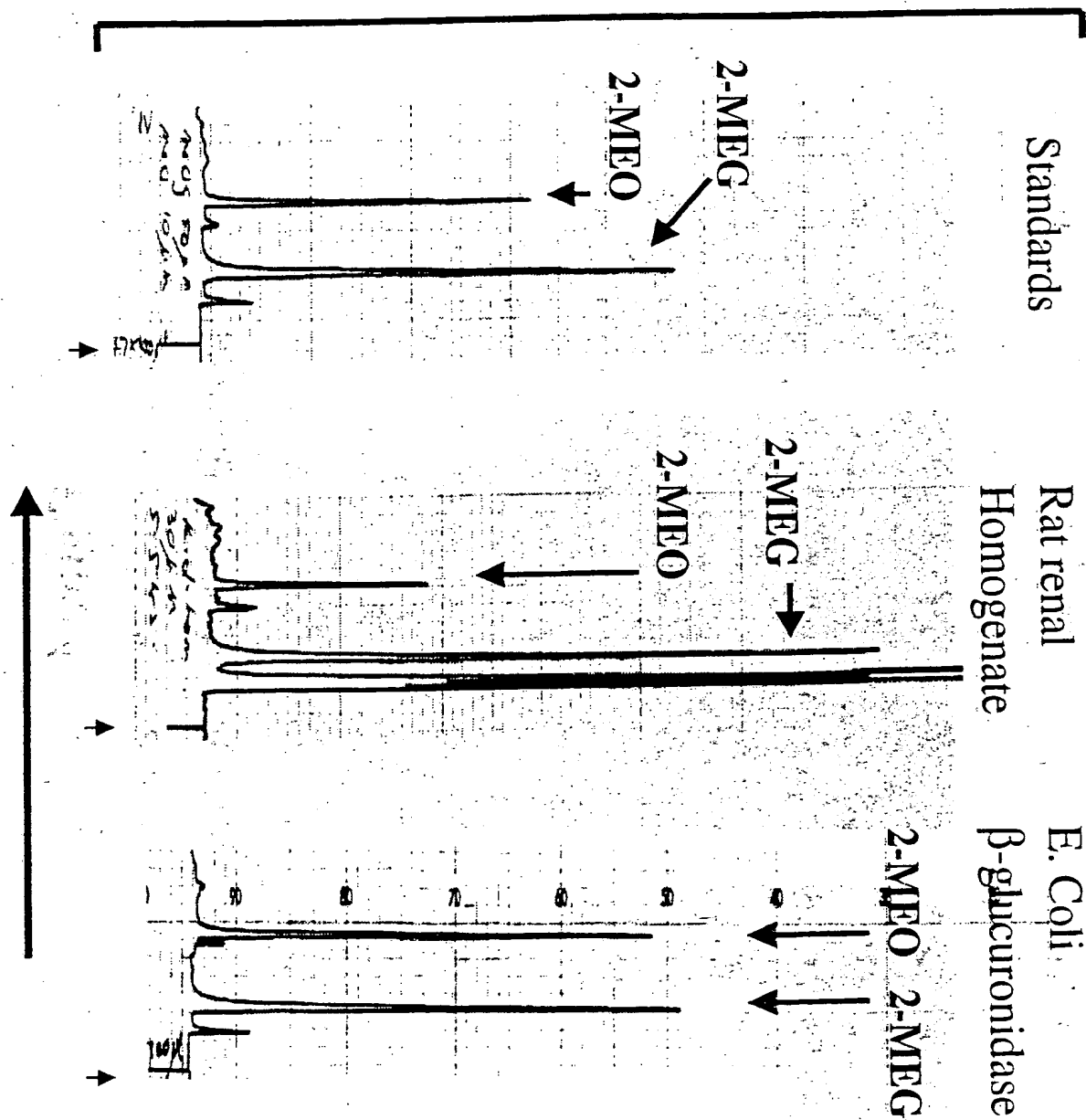
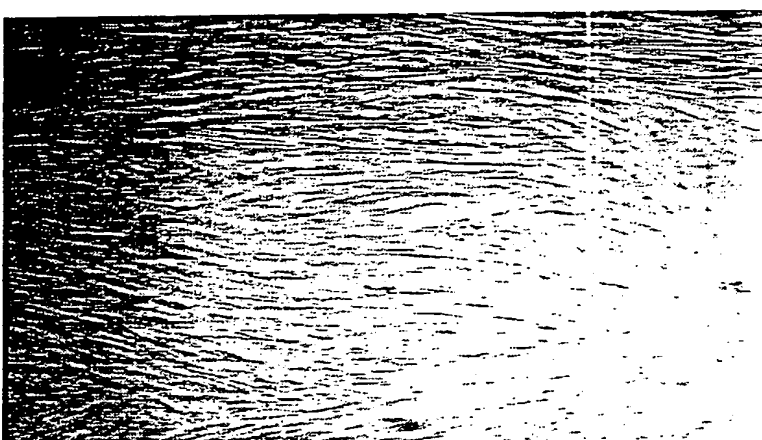
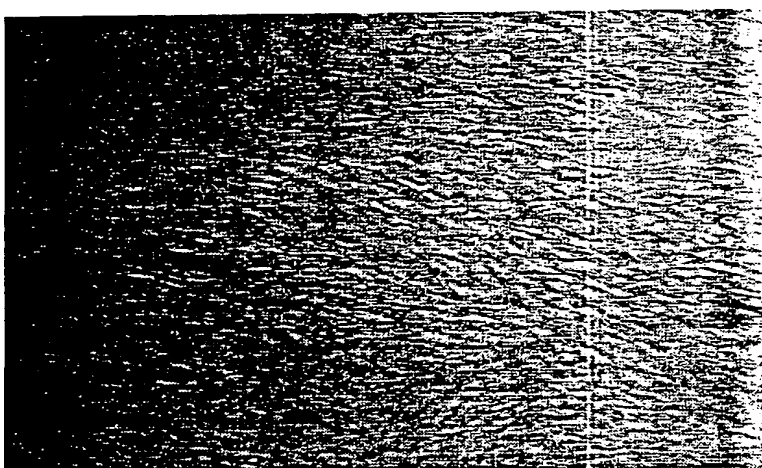
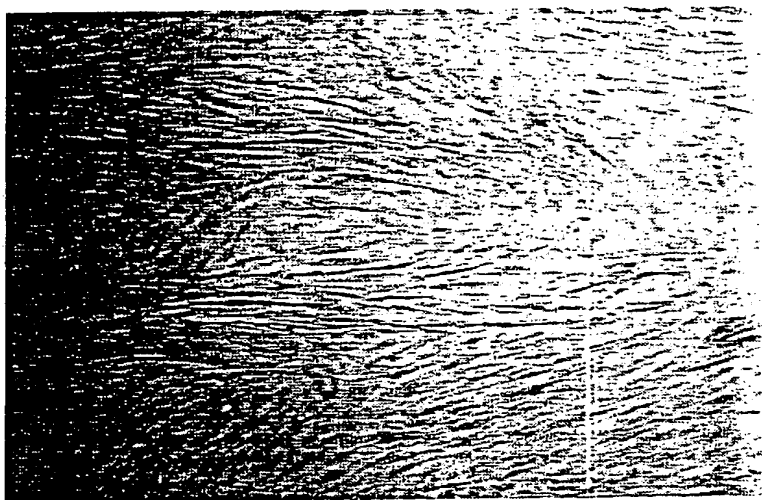


Figure 4  
Reduced airway smooth muscle shape-change induced  
by  $17\beta$ -D-glucuronide-2-Methoxyestradiol (2-MEG)

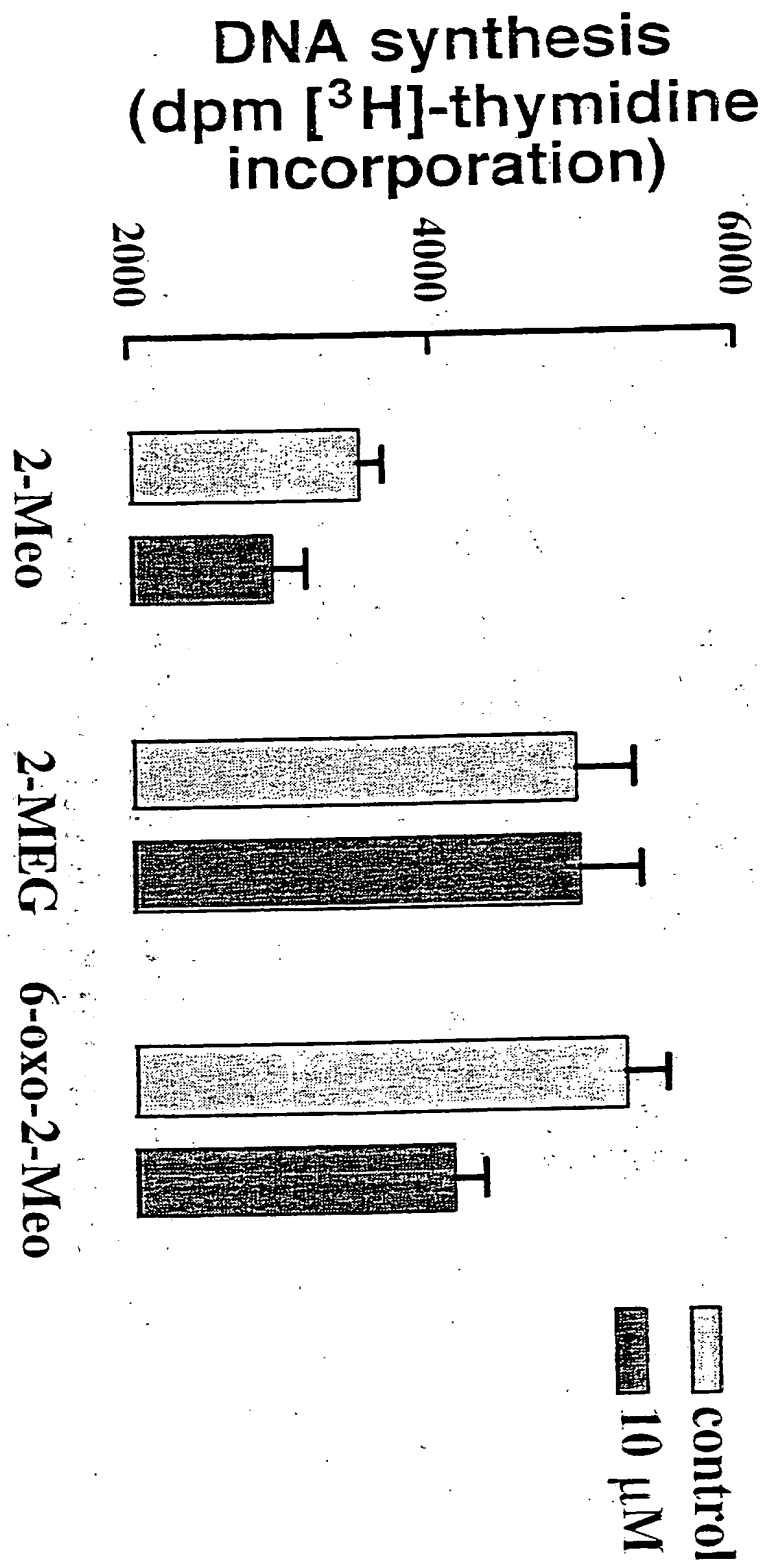


FCS alone

FCS +  
2-MEO 10  $\mu$ M

FCS +  
2-MEG 10  $\mu$ M

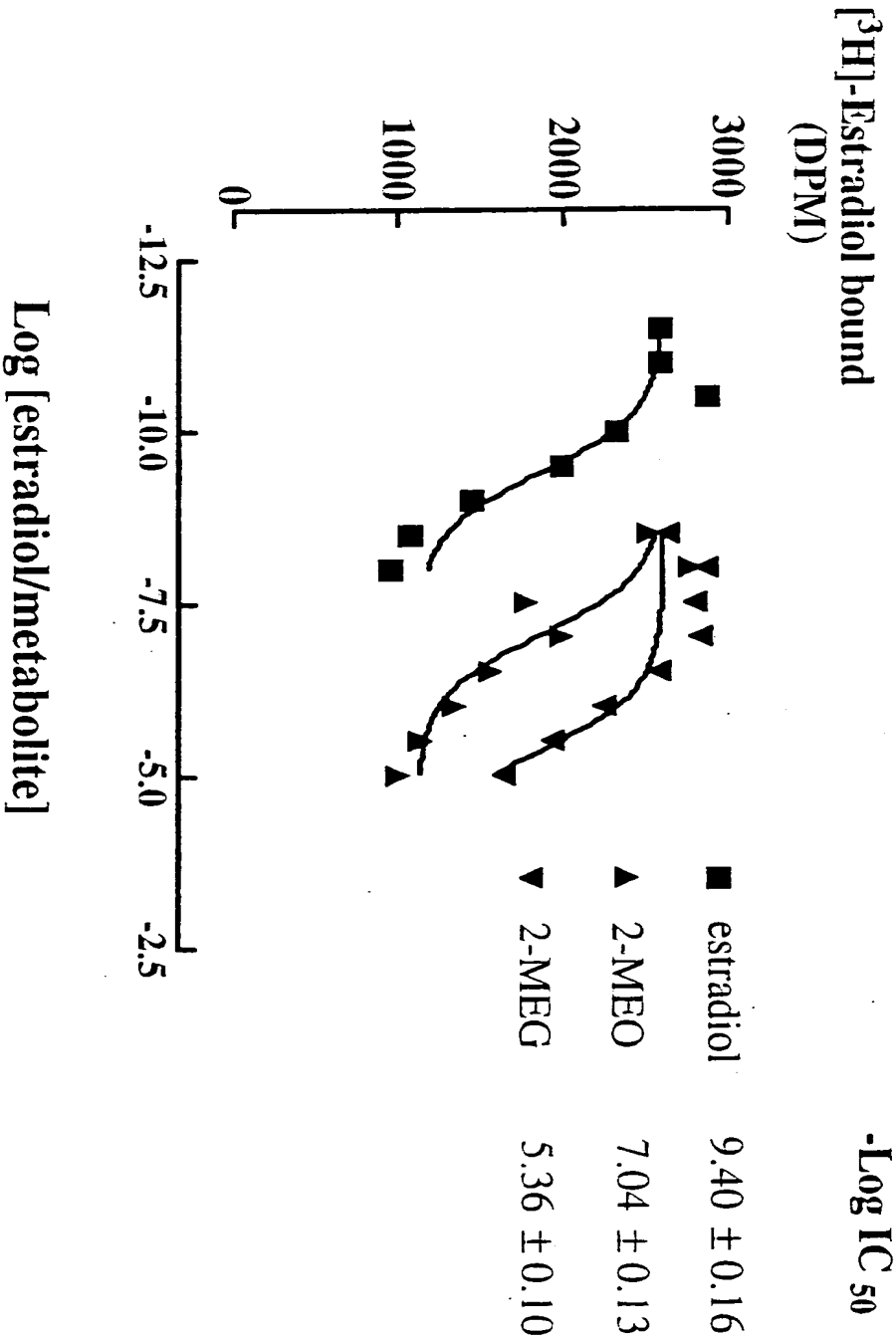
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**Figure 5**  
**Effects of 2-Meo and**  
**analogues on DNA synthesis in**  
**the human epithelial cell line**  
**A549**

Figure 6

Reduced estrogen receptor affinity  
of 17 $\beta$ -D-glucuronide-2-Methoxyestradiol (2-MEG)



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/AU00/01244

|  |  |  |  |  |  |
|--|--|--|--|--|--|
| <b>A. CLASSIFICATION OF SUBJECT MATTER</b>   |  |  |  |  |  |
| Int. Cl. <sup>7</sup> : C07J 1/00; A61K 47/36, 47/42, 47/48; A61P 9/00, 35/00.   |  |  |  |  |  |
| According to International Patent Classification (IPC) or to both national classification and IPC  |  |  |  |  |  |
| <b>B. FIELDS SEARCHED</b>  |  |  |  |  |  |
| Minimum documentation searched (classification system followed by classification symbols)  |  |  |  |  |  |
| Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  |  |  |  |  |  |
| Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)<br>Database: STN, File: CA, substructure: steroid skeleton plus 5 or 6 membered sugar and 2-methoxy; keywords: estra and diene, estradiol, acet?, hydrazon?, and 2-methoxy.   |  |  |  |  |  |
| <b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>  |  |  |  |  |  |
| Category*  | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No.  |  |  |  |
| X<br>Y   | Chem. Pharm. Bull. 1982, vol. 30(9), pages 3231-3238. K. Watanabe et al, "Preparation and high-performance liquid chromatographic determination of guaiacol estrogen 17 $\beta$ -conjugates: The enzymatic O-methylation products of 2-hydroxyestradiol 17 $\beta$ -conjugates."<br>See page 3232 Chart 1 and especially compound 9.   | 1-17, 20-24, 32-34<br>26-31  |  |  |  |
| X<br>Y   | Steroids, 1997, vol. 62, pages 750-761. A. C. Stalford et al, "The metabolism of 16-fluoroestradiols in vivo: Chemical strategies for restricting the oxidative biotransformations of an estrogen-receptor imaging agent."<br>See figure 10b and<br>Registry Number 202397-96-4.   | 1-17, 21-24, 34<br>26-31   |  |  |  |
| <input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C <input checked="" type="checkbox"/> See patent family annex  |  |  |  |  |  |
| <table style="width: 100%; border: none;"> <tr> <td style="width: 33%; vertical-align: top;">           * Special categories of cited documents:<br/>           "A" document defining the general state of the art which is not considered to be of particular relevance<br/>           "E" earlier application or patent but published on or after the international filing date<br/>           "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)<br/>           "O" document referring to an oral disclosure, use, exhibition or other means<br/>           "P" document published prior to the international filing date but later than the priority date claimed         </td> <td style="width: 33%; vertical-align: top;">           "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention<br/>           "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone<br/>           "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art<br/>           "&amp;" document member of the same patent family         </td> <td style="width: 33%;"></td> </tr> </table> |  |  | * Special categories of cited documents:<br>"A" document defining the general state of the art which is not considered to be of particular relevance<br>"E" earlier application or patent but published on or after the international filing date<br>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)<br>"O" document referring to an oral disclosure, use, exhibition or other means<br>"P" document published prior to the international filing date but later than the priority date claimed | "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention<br>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone<br>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art<br>"&" document member of the same patent family |  |
| * Special categories of cited documents:<br>"A" document defining the general state of the art which is not considered to be of particular relevance<br>"E" earlier application or patent but published on or after the international filing date<br>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)<br>"O" document referring to an oral disclosure, use, exhibition or other means<br>"P" document published prior to the international filing date but later than the priority date claimed   | "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention<br>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone<br>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art<br>"&" document member of the same patent family |  |  |  |  |
| Date of the actual completion of the international search<br>7 December 2000   |  | Date of mailing of the international search report<br>12 DEC 2000        |  |  |  |
| Name and mailing address of the ISA/AU<br><br>AUSTRALIAN PATENT OFFICE<br>PO BOX 200, WODEN ACT 2606, AUSTRALIA<br>E-mail address: pct@ipaustrialia.gov.au<br>Facsimile No. (02) 6285 3929   |  | Authorized officer<br><br>FRANCES RODEN<br>Telephone No : (02) 6283 2239 |  |  |  |

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| C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT |  |                                |
|---|--|--------------------------------|
| Category*   | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No.          |
| X<br>Y  | Chemical Abstract 124:4208<br>& Bunseki Kagaku, 1995, vol. 44(10), pages 793-796. K. Takanashi et al, "HPLC separation of ring D-conjugated estrogens."<br>See abstract.   | 1-17, 20-24,<br>34<br>26-31    |
| X<br>Y  | Steroids, 1990, vol. 55(3), pages 128-132. T. Ohkubo et al, "Synthesis of 2-hydroxyestriol monoglucuronides and monosulfates."<br>See especially page 131.   | 1-17, 21-24,<br>32-34<br>26-31 |
| X<br>Y  | Acta Endocrinologica, 1982, vol. 100, pages 57-62. M. Holler et al, "Simultaneous perfusion of [14- <sup>14</sup> C]oestriol and [6,9- <sup>3</sup> H <sub>2</sub> ]oestriol 16 $\alpha$ -monoglucuronide through the isolated rat liver."<br>See especially page 57 column 1 line 28, page 58 column 1 line 12 and page 60, and Registry Number 82073-95-8. | 1-17, 21-24<br>26-31           |
| X<br>Y  | Chem. Pharm. Bull. 1977, vol. 25(5), pages 942-948. T. Nambara et al, "Biliary conjugated metabolites of estriol in the rat."<br>See especially page 943 paragraph 3, page 944 compounds V-XI and 1-6.   | 1-17, 21-24<br>26-31           |
| X<br>Y  | Journal of Steroid Biochemistry, 1976, vol. 7, pages 749-756. B. Spiegelhalter et al, "Mass-spectrometry of steroid glucuronides."<br>See especially page 756 table 2 and Registry Number 52718-01-1.  | 1-17, 21-24<br>26-31           |
| X<br>Y  | Chem. Pharm. Bull. 1976, vol. 24(3), pages 421-426. T. Nambara et al, "Syntheses of methoxyestrogen glucuronide acetate-methyl esters."<br>See especially page 422 compounds.  | 1-17, 21-24,<br>32-34<br>26-31 |
| X<br>Y  | Hoppe-Seyler's Z. Physiol. Chem, 1974, vol. 355, pages 490-494. G. Rohle et al, "Synthesis of glucuronides of 2-hydroxylated oestrogens and their methyl ethers."<br>See especially page 491 and Registry Numbers 52717-98-3 and 52718-01-1.   | 1-17, 20-24,<br>32-34<br>26-31 |
| X<br>Y  | WO 95/04535 (THE CHILDREN'S MEDICAL CENTER CORPORATION), 16 February 1995.<br>See entire document.   | 10-15, 20<br>26-31             |

Form PCT/ISA/210 (continuation of Box C) (July 1998)

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/AU00/01244

| C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT |  |                       |
|--|--|-----------------------|
| Category*  | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
| X  | Chemical Abstract 97:163313<br>& Hoppe-Seyler's Z. Physiol. Chem, 1982, vol. 363(7), pages 737-744. D. Berg et al, "Synthesis of immunogenic C-6 derivatives of 2-methoxyestrone and 2-methoxy-17.beta.-estradiol and characterization of the corresponding antiscrums."<br>See abstract and Registry Numbers 83274-89-9 and 69540-62-1P | 10-15, 20             |
| X  | Chemical Abstract 90:121859<br>& Chem. Pharm. Bull, 1978, vol. 26(11), pages 3567-3571. A. Nakagawa et al, "Clinical analysis on steroids. Part V. Synthesis of 2,3,17.beta.-trihydroxyestra-1,3,5(10)-trien-6-one and its related compounds."<br>See abstract and Registry Number 7291-57-8   | 10-15, 20             |

# INTERNATIONAL SEARCH REPORT

International application No  
PCT/AU00/01244

## Box I Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos : 29-31  
because they relate to subject matter not required to be searched by this Authority, namely:  
These claims are to a method of treatment. Under rule 67.1 of the PCT this is excluded subject matter. However, the search has been carried out based on the effects of the compound or functionally active analogue or derivative thereof.
2. ☒ Claims Nos : 1-19 and 21-34  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  
A full search was not possible on economic grounds. The scope of the claims is so broad that a meaningful search was not possible. The search was therefore limited to the examples described in the specification and only estradiol compounds/conjugates containing a 2-methoxy group and a sugar were searched. Claims 18,19 and 25 were therefore not searched. Claim 20 was searched in full.
3. ☐ Claims Nos :  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

## Box II Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.



# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.  
PCT/AU00/01244

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent Document Cited in Search Report |          |    |          | Patent Family Member |         |    |              |
|--|----------|----|----------|----------------------|---------|----|--------------|
| WO                                     | 95/04535 | AU | 74509/94 | CA                   | 2168850 | EP | 713393       |
|  |          | JP | 9501433  | US                   | 5504074 | US | 5661143      |
|  |          | US | 5892069  |                      |         |    |              |
|  |          |    |          |                      |         |    | END OF ANNEX |

